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THE ANTIGENIC COMPOSITION OF BUFFALO SEMEN

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INTRODUCTION

The antigenic property of spermatozoa, first demonstrated by Landsteiner (1899) working with bull spermatozoa, has since been confirmed by Metchnikoff (1900 a,b,) and many others. The earlier work has been reviewed by Smith (1950). Henle, Henle and Chambers (1938) were able to show that antigenic differences could be demonstrated between the head and the tail. Weil, Kotesevalov and Wilson (1956) have made a study of the antigens in human semen. They were unable, however, to find out whether spermatozoa have antigens different from seminal plasma. Though it has been known that mammalian spermatozoa are antigenic, so far the antigenic composition of mammalian semen has not been demonstrated. It is also not known whether there are common antigens between blood serum and some of the secretions of the reproductive tract.

The object of the following studies was to study the antigenic composition of the buffalo-bull seminal plasma, spermatozoa, prostatic secretion, vesicular secretion, ampullar secretion and cervical mucus, using the Ouchterlony gel diffusion technique. Attempts were also made to detect common or cross-reacting antigens between these secretions and blood serum.

MATERIALS AND METHODS

Buffalo semen, prostatic fluid and cervical secretions were obtained from the Bombay Government Milk Colony at Aarey. Prostatic secretions were collected by massaging the prostate per rectum. Blood was obtained from the jugular vein of a healthy animal. Seminal vesicular fluid, ampullar fluid and epididymal spermatozoa were collected from a bull immediately after it was slaughtered.

Specific antibodies to buffalo semen were prepared from the blood of rabbits immunised with semen frozen and thawed six times. Freezing and thawing was carried out with the hope of damaging the spermatozoa and thus obtaining more of the spermatozoal antigens. Injections of semen were given subcutaneously once a week for at least 6 to 7 weeks before the rabbits were bled. Serum was preserved in the cold after the addition of merthiolate in a concentration of 1:10,000. About 10 cc. of blood was taken from the rabbits every week and they were injected with 0·5 cc. semen soon after.

To obtain sperm antigens, spermatozoa were washed 6 times with saline by slow centrifugation at 1000 r.p.m. for 15 minutes and suspended in a volume of saline equal to the volume of seminal plasma removed. The suspension was repeatedly freeze-thawed at least 6 times in an attempt to extract sperm antigens.

The gel diffusion technique of Ouchterlony (1949) was used to determine the common and cross-reacting antigens. To a 1 per cent solution of agar (Difco) filtered through Whatman No. 1 filter paper, 0·1 cc. of a 1 per cent solution of eosin was added to give a pale pinkish colour. The colour facilitated the reading of the precipitin lines. A volume of 20 cc. was poured into petri dishes with a diameter of 10 cm. After the agar had set, stainless steel penicillin assay cups warmed slightly over a flame were inserted so that the cups partially penetrated the agar. Usually one cup was placed in the centre and six others in a circle of 2·2 cm. from each other and also from the central cup. The central cup was filled with antiserum and the surrounding cups with different antigens.

In order to detect small amounts of antibodies, the highly sensitive haemagglutination technique of Boyden (1951) and Stavitsky (1954) was used. The test had to be slightly modified as the erythrocytes were coated with the antibody instead of the antigen. The suspension of tannic acid-coated erythrocytes was prepared according to the method of Stavitsky (1954). Rabbit antiserum to buffalo semen was inactivated at 56°C. and absorbed with sheep erythrocytes to remove non-specific agglutinins. To 4 cc. of the buffered saline (pH 6·4) was added 1 cc. of the heat-inactivated and absorbed antibody, and 1 cc. of the tannic acid-coated sheep erythrocytes and the mixture kept at the room temperature for 30 minutes. The erythrocytes were centrifuged, washed once with the buffered saline and resuspended in 1 cc. of the same buffer. A 0·05 cc. volume of the suspension of antibody coated erythrocytes was measured out into Kahn tubes containing 0·5 cc. of a serial dilution of the antigen under test. The contents of the tubes were mixed and they were allowed to stand at room temperature for 3 to 6 hours before they were observed for agglutination. The highest dilution of the antigen giving a positive agglutination and the degree of reaction as described by Stavitsky (1954) were also noted. The following controls were also put up at the same time: 1) antigen under test and tannic acid-treated erythrocytes, 2) tannic acid-treated erythrocytes suspended in diluent, and 3) tannic acid-treated erythrocytes coated with antibody and suspended in diluent.

The fructose content of seminal plasma and the other secretions was estimated by the method of Mann (1948).

Citric acid was estimated by the method of Saffron and Denstedt (1948).

RESULTS

The fructose and citric acid content of prostatic secretion and seminal vesicular secretion was estimated to ascertain whether these secretions were from the respective glands since it is known that the prostatic secretion does not contain either fructose or citric acid. Table I gives the values obtained. The figures in parenthesis are the values for bull seminal plasma, prostatic secretion and seminal vesicular secretion as given by Mann (1954).

The results show that both prostatic secretion and vesicular secretion were from the respective glands since prostatic secretion gave a value of only 10 mg. fructose/100 cc. compared to the 972 mg./100 cc. of vesicular secretion. The small reading for prostatic

TABLE 1

FRUCTOSE, AND CITRIC ACID CONTENT OF BUFFALO BULL SEMINAL PLASMA, PROSTATIC SECRETION AND VESICULAR SECRETION

Sample tested	*Fructose content in mg/100 cc.*	*Citric acid content in mg/100 cc.*
Seminal plasma	464 mg. (280—770 mg)	826 mg. (340—1150 mg)
Prostatic secretion	10 mg.	nil.
Vesicular secretion	972 mg. (970 mg)	280 mg. (670 mg)

secretion might have been due to traces of other reducing substances: there was no trace of citric acid.

The results of a series of experiments using the Ouchterlony gel diffusion technique are summarized in Tables 2 and 3 while Figs 1 to 8 give the photographs of the agar plates.

The following points must be considered in interpreting the results obtained. The number of precipitin lines indicate only the minimum number of antigens in the system reacting with the corresponding antibodies in the test serum. If the concentration of any one of the antigens or its corresponding antibody is below the level necessary to form a precipitate, then no precipitin line will be formed in the agar plate. It is also common experience that the numbers of lines formed with diluted antigens and antibodies are less than those formed with the concentrated ones. Moreover, it is likely that two or more precipitin lines may merge and look like one if the antigen-antibody ratios of the systems coincide. One disadvantage of the gel diffusion technique is that only the water soluble antigens are accounted for. Hence, some of the insoluble antigens which may be present in spermatozoa will not be detected by this technique.

Whenever the lines formed with the same antiserum and the different antigens merge in the plates, it is an indication of indentity of antigens or very close relationship (Korngold and Van Leeuwen, 1957). In the various experiments such merging was seen between seminal plasma and prostatic and vesicular secretion, cervical mucus and blood serum (Fig. 1, 2 and 4). If there is partial merging and a spur it is usually taken to mean a cross reaction and not an identity of antigens. Blood serum and prostatic secretion showed such cross reaction with spermatozoa when antibody to semen was used (Figs. 3 and 4).

Table 2 summarizes the results where antiserum to whole semen was used. A number of plates were put up where the antigens in adjacent cups were varied to enable the detection of common or cross-reacting antigens. Whole semen and seminal plasma have shown 16 antigens whereas freeze-thawed spermatozoa have shown

TABLE 2

GEL DIFFUSION TECHNIQUE USING ANTIBODY TO BUFFALO SEMEN

Antigens used	*Number of antigen-antibody precipitin lines formed*			
	Strong intense lines	*Lines of moderate intensity*	*Faint lines*	*Total number of lines*
Semen	2	3	11	16
Freeze-thawed semen	3	3	11	17
Seminal plasma	2	3	11	16
Freeze-thawed spermatozoa	1	2	4	7
Freeze-thawed epididymal spermatozoa	2	2	3	7
Prostatic secretion	—	2	2	4
Vesicular secretion	1	3	1	5
Ampullar secretion	1	1	1	3
Cervical mucus	—	2	2	4
Blood serum	—	2	4	6

7. Prostatic secretion has shown 4 common to seminal plasma. Vesicular secretion has shown 5 and ampullar secretion 3 antigens. Vesicular secretion has shown 2 antigens in common with spermatozoa and two in common with ampullar secretion. All the four antigens of prostatic secretion are common to blood serum (Figs. 2 and 4) whereas seminal vesicular secretion has two antigens which do not seem to be common to blood serum. Semen has 6 antigens common to blood serum. There is one antigen which is common to seminal plasma, cervical mucus, freeze-thawed whole semen, freeze-thawed sperm and blood serum.

The results of the experiments outlined above suggested that spermatozoa have some antigens different from seminal plasma. To prove this supposition the inhibition test of Bjorklund (1952) was carried out. Equal amounts of seminal plasma and rabbit antiserum to whole semen were mixed and incubated at 37°C for 30 minutes so that the antibodies to seminal plasma antigens could be neutralized. The absorbed antiserum was put in the central cup of an agar plate and round it were placed in order freeze-thawed sperm, blood serum, cervical mucus, freeze-thawed semen, seminal plasma, and unabsorbed antiserum (Fig. 7). The position of the unabsorbed antiserum was such that it was between freeze-thawed sperm and seminal plasma. After a week, precipitin lines began to develop and the results are summarized in Table 3.

The results indicate that buffalo spermatozoa have at least 3 antigens not common either to seminal plasma or to blood serum and that blood serum has antigens common to seminal plasma. Seminal plasma has not shown any precipitin lines with absorbed antiserum whereas it has shown 16 lines with unabsorbed antiserum. Freeze-thawed spermatozoa have shown 7 lines with the unabsorbed antiserum. Three lines of medium intensity were seen between absorbed and unabsorbed antiserum which means that in the absorbed antiserum the seminal plasma was in excess of the amount needed for neutralization.

TABLE 3

GEL DIFFUSION TECHNIQUE USING ANTISERUM TO FREEZE-THAWED SEMEN ABSORBED BY SEMINAL PLASMA

Antigens used	*Precipitin lines seen with absorbed antiserum*
Seminal plasma	Nil
Freeze-thawed semen	1 line of medium intensity 2 faint lines
Freeze-thawed spermatozoa	3 faint lines
Blood serum	Nil
Cervical mucus	2 faint lines
Antiserum to semen	3 faint lines

The observations reported so far have shown that spermatozoa have at least seven antigens whereas seminal plasma has at least 16. As already reported earlier, Weil, *et al.* (1956) working with human semen have reported that seminal plasma and spermatozoa have the same number of antigens. It was of interest, therefore, to find out whether the 7 precipitin lines invariably observed with washed and freeze-thawed spermatozoa and antiserum to semen could also be observed with epididymal spermatozoa.

An experiment was carried out using epididymal and washed ejaculated spermatozoa. Epididymal spermatozoa were scraped out of the epididymis after it was slit open. The spermatozoa were suspended in 0·85 per cent saline and thoroughly mixed. The suspension was centrifuged at 1500 r.p.m. for 20 minutes. The spermatozoa were washed once in order to remove the other secretions of the epididymis, freeze-thawed and used as the antigen. Both epididymal spermatozoa and ejaculated spermatozoa have shown the same number of antigens. These results suggest both epididymal and ejaculated spermatozoa have at least 7 antigens, 3 of which are not common to either seminal plasma or blood serum. Both ejaculated and epididymal spermatozoa have always shown antigens which are not common to prostatic secretion.

Effect of Freezing and Thawing on Spermatozoa

Experiments were carried out to find out whether freezing and thawing of spermatozoa would result in larger amounts of antigen being released from the spermatozoa into the surrounding medium.

A 4 cc. volume of semen, pipetted into a 15 cc. tube, was centrifuged at 1500 r.p.m. for 25 minutes and the plasma was removed. The sedimented spermatozoa were then washed twice in about 5 cc. of saline. The washed spermatozoa were suspended in saline and the volume was made up to the original volume of semen.

This suspension was then divided into two portions: one was kept at 5°C and the other portion was freeze-thawed repeatedly 6 times over a period of about 6 to 8 hours. Both samples were centrifuged at 1500 r.p.m. for 20 minutes. The supernatants were carefully removed to estimate the nitrogen content. The gel diffusion test and the haemagglutination test were carried out.

Nitrogen values were determined by the Kjeldahl procedure. The supernatant from the freeze-thawed spermatozoa had a value of 23 mg N/cc. while the supernatant of the semen sample kept at 5°C had 10·5 mg N/cc. indicating that more of the spermatozoal antigens were released by repeated freezing and thawing. In the gel diffusion test the supernatant from the freeze-thawed spermatozoa showed one medium and two faint precipitin lines whereas the other supernatant did not show any. It seems very likely that the concentration of the antigens in the sample kept at 5°C was insufficient for the formation of precipitin lines.

The haemagglutination test was carried out by coating tannic acid-treated sheep erythrocytes with rabbit antiserum to buffalo semen. Such antibody coated cells were washed once with buffered saline (pH 6·4) and suspended in buffered saline. Serial dilutions of the two antigens were made and to each was added 0·05 cc. of the antibody-coated sheep erythrocytes. The highest dilution showing agglutination with the supernatant from the freeze-thawed spermatozoa was 1:32 while the supernatant from the spermatozoa kept at 5°C showed agglutination with the undiluted sample only.

DISCUSSION

In the gel diffusion test buffalo seminal plasma has shown the presence of 16 and spermatozoa 3 antigens which are not common to seminal plasma. However, whole semen has shown only 17 while it should have shown 19 antigens. The reasons for this may be due, as suggested above, to the superimposition of some antigen-antibody precipitin lines.

Washed and freeze-thawed spermatozoa have shown the presence of 7 antigens of which 3 are not present in seminal plasma. It is therefore apparent that 4 antigens are common to both spermatozoa and seminal plasma and this may be due to the permeability of the spermatozoal cell wall. Mann (1949) has commented on the remarkable permeability of the spermcell and according to him, this property explains why a number of intermediary enzymes, particularly those concerned with respiration, can be demonstrated directly in intact spermatozoa without having to lyse the cells which is a prerequisite to the study of such enzymes in other body tissue. It has also been shown by Hetcher (1947) and Mann (1949) that spermatozoa easily release hyaluronidase into the surrounding medium. Mann and Lutwak-Mann (1955) have shown that cytochrome C and intracellular proteins of ram spermatozoa are released when they are subjected to the shock of sudden lowering of temperature. Sheth and Rao (1959) have observed that the succinic dehydrogenase activity of human semen goes up in the cold when semen is stored at 5°C. The release of 10·5 mg N/cc. in the supernatant of the spermatozoal suspension kept at 5°C is probably due to the temperature shock. The effect of such a shock is apparently far greater during repeated freezing and thawing.

Another reason why some of the spermatozoal material is released into the seminal plasma could be due to some mechanical damage during centrifugation. According to Mann (1954) spermatozoa are highly vulnerable though the damage may not be apparent. Seminal plasma may also possess spermatozoal antigens because some of them could be products of metabolic activity of the spermatozoa.

Of the 16 antigens in buffalo seminal plasma, at least 4 are contributed by the prostate, 5 by the seminal vesicles and 3 by the ampulla. Seminal vesicular secretion has shown 2 antigens in common with spermatozoa and 2 antigens in common with ampullar secretion. None of the antigens of the prostate are common to either spermatozoa or vesicular secretion. It is very clearly seen in Fig. 3 that antigens of the prostatic secretion and those of spermatozoa are neither common nor reacting. The occurrence of common antigens between seminal plasma and blood serum on the one hand and between cervical mucus and blood serum on the other suggests that the gamma globulin of blood may be present in these secretions. If this were true it would mean that any antibody circulating in the blood would be present in these secretions.

It was not possible to immunize a female-buffalo to demonstrate the occurrence of gamma globulin in the cervical mucus, but we have attempted to demonstrate the occurrence of antibody in the cervical mucus of a mare which was hyperimmunized with diphtheria toxin. After about 4 days antigen—antibody precipitin lines developed between the toxin and serum and between the toxin and mucus. These lines later merged, thereby showing their identity (Fig. 8).

Rao and Sadri (1959) working with human semen have reported that seminal plasma and cervical mucus have antigens common to blood serum. By making use of the sensitive haemagglutination technique they have further shown that in some cases of infertility auto-antibodies to spermatozoa were present in the seminal plasma and blood serum. The presence of auto-antibodies to spermatozoa in the blood serum is not surprising since it has been shown that there are antigens common to blood serum and seminal plasma. The occurrence of antibodies in seminal plasma can be explained only on the basis that spermatozoa contain some antigens not common to seminal plasma.

CONCLUSIONS

The antigenic composition of buffalo semen was studied using the Ouchterlony gel diffusion technique.

Buffalo seminal plasma contains at least 16 antigens and spermatozoa at least 7, of which 4 are common to seminal plasma.

Prostatic secretion shows the presence of 4 antigens common to semen and to blood serum. Seminal vesicular secretion and ampullar secretion show 5 and 3 antigens respectively in common with semen. Two of the antigens of the seminal vesicular secretion, are common to spermatozoa and 2 to ampullar secretion.

Six antigens of buffalo blood serum are common to seminal plasma and 4 to cervical mucus. The results indicate that antibodies circulating in the blood could also be present in cervical mucus.

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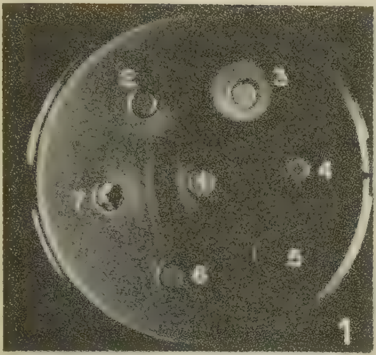

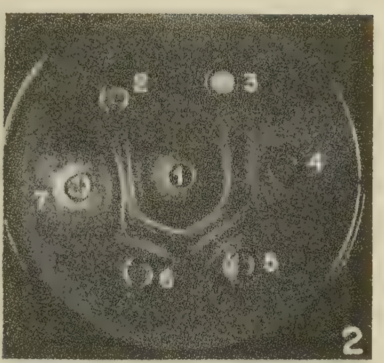

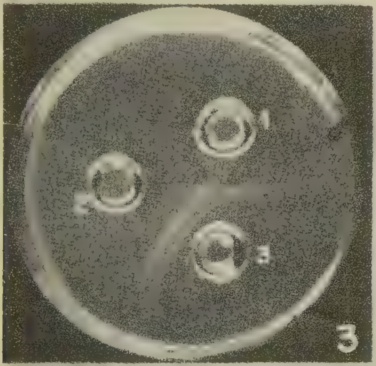

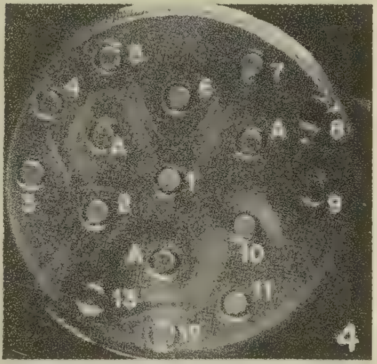

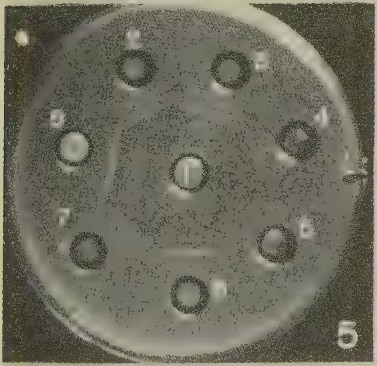

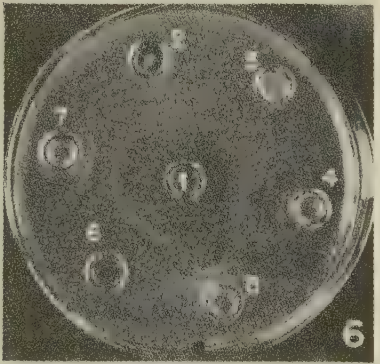

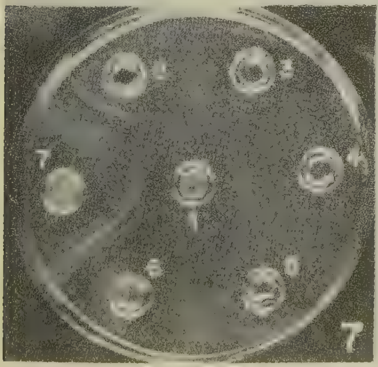

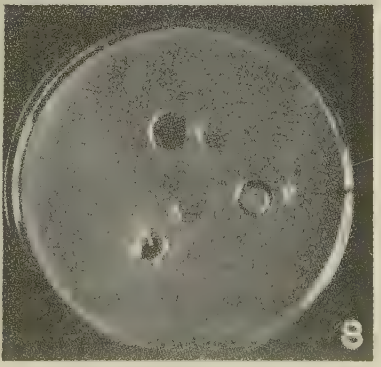

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LEGENDS TO ILLUSTRATIONS

Fig. 1
1. Antiserum to freeze-thawed buffalo semen
2. Washed freeze-thawed spermatozoa
3. Blood serum
4. Seminal plasma
5. Cervical mucus
6. Prostatic fluid
7. Freeze-thawed semen

Fig. 2.
1. Antiserum to freeze-thawed buffalo semen
2. Blood serum
3. Prostatic fluid
4. Whole semen
5. Freeze-thawed semen
6. Washed freeze-thawed spermatozoa
7. Seminal plasma

Fig 3.
1. Prostatic fluid
2. Freeze-thawed epididymal spermatozoa
3. Rabbit antiserum to freeze-thawed semen

Fig. 4.
A. Antiserum to freeze-thawed buffalo semen.
1. Ampullar fluid
2. Vesicular fluid
3. Freeze-thawed semen
4. Freeze-thawed spermatoxoa
5. Freeze-thawed epididymal spermatozoa
6. Seminal plasma
7. Vesicular fluid
8. Freeze-thawed epididymal spermatozoa
9. Blood serum
10. Prostatic fluid
11. Seminal plasma
12. Semen
13. Blood serum

Fig. 5.
1. Antiserum to freeze-thawed buffalo semen
2. Semen
3. Freeze-thawed semen
4. Freeze-thawed epididymal spermatozoa
5. Washed epididymal spermatozoa
6. Washed freeze-thawed spermatozoa
7. Washed spermatozoa
8. Seminal plasma

Fig. 6.
1. Antiserum to freeze-thawed semen
2. Seminal plasma
3. Washed and freeze-thawed epididymal spermatozoa
4. Washed and freeze-thawed spermatozoa
5. Washed spermatozoa
6. Washed epididymal spermatozoa
7. Semen

Fig. 7.
1. Antiserum to Freeze-thawed buffalo semen absorbed with seminal plasma
2. Freeze-thawed washed spermatozoa
3. Cervical mucus
4. Freeze-thawed semen
5. Blood serum
6. Seminal plasma
7. Antiserum to freeze-thawed semen

Fig. 8.
1. Diphtheria toxin
2. Diphtheria antitoxin
3. Cervical mucus from a hyperimmunized mare

THE OCCURRENCE OF DISSOCIATED STRAINS OF *BRUCELLA MELITENSIS* IN THE MILK OF GOATS IN MALTA

By

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INTRODUCTION

In the routine typing of *Br. melitensis* isolated from goats' milk in Malta, it was found that 11 of 46 strains gave entirely negative reactions to agglutination tests in which both mono-specific *Br. abortus* and mono-specific *Br. melitensis* sera were used: in all other respects these strains appeared to be typical *Br. melitensis*. All the strains were isolated on albimi agar containing antibiotics and the first subculture, made by picking a single colony on to serum-dextrose agar, was used in the tests.

Some further work has been undertaken on (1) the frequency of occurrence of dissociated strains of *Br. melitensis* in the milk of a relatively small number of Maltese goats, (2) the virulence of such strains for rabbits and (3) some serological investigations, including a study of the antigenic structure of two dissociated strains.

MATERIALS AND METHODS

Samples of milk from individual goats in known infected herds were taken into one ounce screw-capped bottles and subjected to stained antigen agglutination tests. The antigen was the haematoxylin-stained ring test antigen obtained from Weybridge. Two drops of antigen were shaken up in 1 ml. of milk, and the results read after one and four hours incubation at 37°C. Agglutination is shown by clumping and deposition of the antigen; only rarely is a ring formed on the surface of the milk. Samples positive to this test were kept for 24 hours in a domestic refrigerator, after which 3 loopfuls of cream and 3 loopfuls of deposit were spread over the surface of each of 3 petri plates. Each petri plate contained a different medium (1) albimi agar with antibiotics (Kuzdas and Morse, 1953 as modified by Renoux, 1954); (2) the same medium with the addition of 1 per cent. dextrose and 5 per cent. horse serum; (3) serum-dextrose agar with the antibiotics as above but without the dye.

Plates on which brucella colonies appeared after 4 days incubation were examined under a stereoscopic microscope for dissociation after being stained with crystal violet by the method described by Spink (1956). Negative plates were incubated for a further 4 days before being discarded. Colonies which had a green-yellow appearance were considered to be smooth, while those appearing purplish-red were considered to be dissociated. One colony of each type was subcultured from each plate on to serum-dextrose agar slopes and subjected to routine typing, i.e. growth on 1/80,000 thionin and 1/50,000 basic fuchsin, H_2S production, agglutination on a slide against 3 types of mono-specific serum "abortus", "melitensis" and "dissociate".

Sera. Well-grown male rabbits were injected intravenously with 1 ml. of a suspension of the strain under test, at a density of approximately 4 on Brown's scale. Bleedings were carried out at intervals.

Antigens for agglutination tests were produced by incubating cultures of the respective strains in Roux flasks of albimi agar for 3 days, suspending in normal saline, heating at 60°C for one hour and finally diluting to a density corresponding to Brown's tube 4. In the case of strain 99, as antigen at a greater density was used, it is about one tube less agglutinable than the others. The standard tube agglutination test was used with doubling dilutions of serum starting at 1/10.

Strains. A number of strains were isolated before the investigation on the frequency in milk had been carried out. The criterion for dissociation was failure to agglutinate either abortus or melitensis mono-specific sera and positive agglutination with 1:2,000 acriflavine on a slide. All strains were isolated from goats' milk. Rabbits were inoculated as follows—

R00 and R01	291 dissociated strain from routine typing.
R1 and R2	436/6 smooth strain from goat 436.
R3 and R4	386 smooth strain from goat 417.
R5 and R6	436/1 dissociated strain from goat 436.

In the next series of strains the criterion for smoothness or dissociation was the colony appearance after staining with crystal violet.

R7	407	smooth strain from herd Q338.
R8	408	mixture 50% smooth and 50% dissociated from single colony from herd Q338.
R9	409	dissociated strain from herd Q338.
R10	426	smooth strain from herd Q261.
R11	427	smooth strain from same goat as 426.
R12	423	dissociated strain from goat No. 7 herd Q49.
R13	424	dissociated strain from goat No. 34 herd Q49.

In addition to the above, control melitensis and abortus sera were obtained from 2 rabbits inoculated with strain 99, a typical, smooth aerobic strain of *Br. abortus*, and from 2 rabbits inoculated with strain 400, a typical smooth strain of *Br. melitensis*.

Autopsy of rabbits R00 to R13 was carried out at intervals of 11 days to 4 weeks after infection except for R9 which was autopsied after 2 months: cultures of various tissues were made on albimi agar containing antibiotics.

Absorption tests. Cells for absorbing serum were obtained by centrifuging a dense suspension of the appropriate strain, harvested from Roux flasks and heated at 60°C. for one hour. Five volumes of serum were thoroughly mixed with one volume of the packed bacterial cells and kept in a bacteriological incubator for 3 hours with frequent shaking after which the cells were separated by centrifugation.

The antigens used for agglutination and the technique of carrying out the tests were as described above. Strain 291 was not satisfactory as an antigen because of instability in saline; instead, strain 266 which was of similar colonial type was used as a homologous antigen. The titres indicated represent 50 per cent. agglutination or more at the dilution stated.

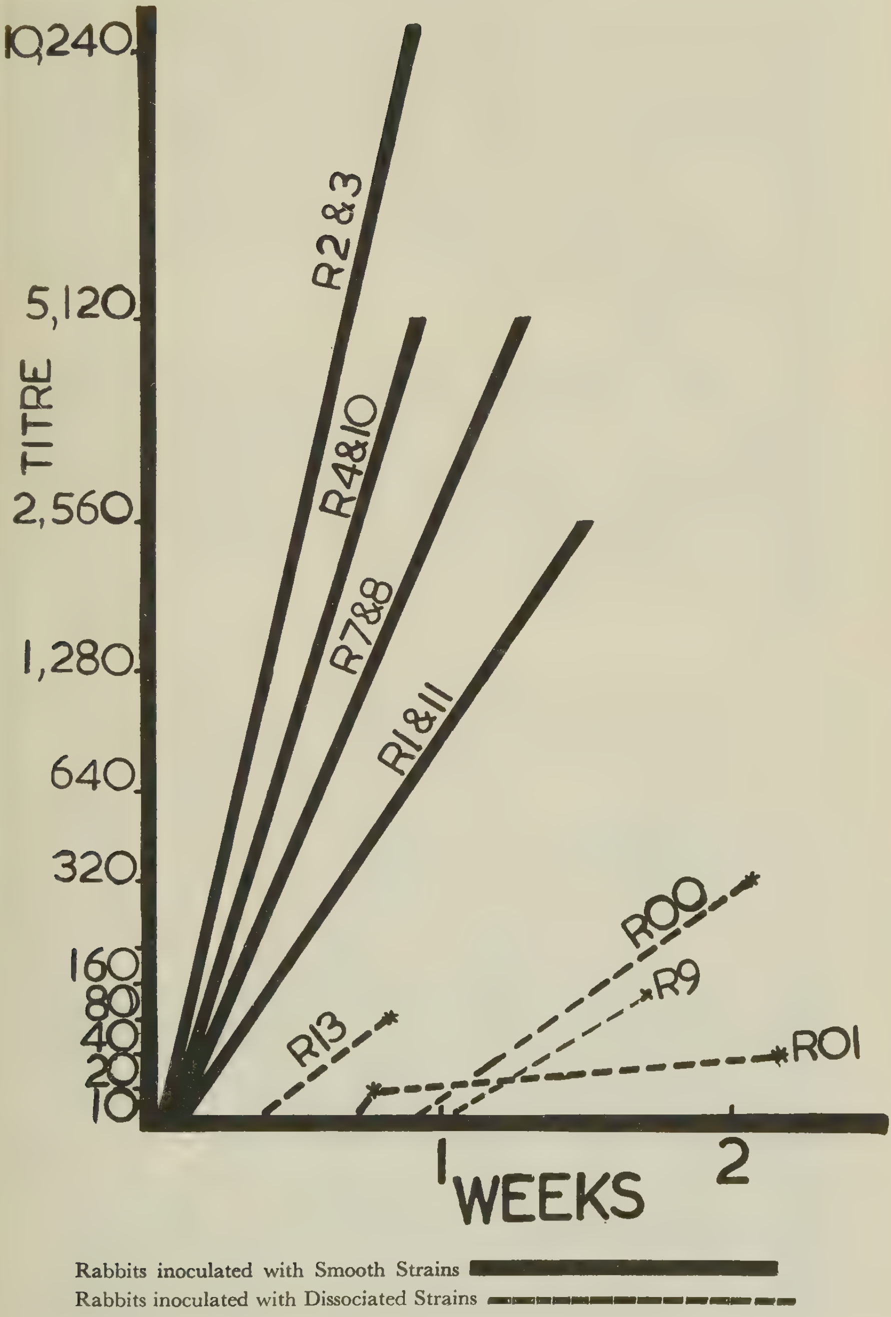
TABLE 1

OCCURRENCE OF DISSOCIATED STRAINS OF *BR. MELITENSIS*

Herd No.	*No. of samples cultured*	*No. of samples positive on culture*	*Degree of infection*	*Samples with only S colonies*	*Samples with all colonies dis-sociated*	*Samples mixed S and dis-sociated*	*% Dis-sociation in mixture*
Q190	16	2	1x, 1xx	2	—	—	
Q338	17	5	1x, 3xxx, 1xxxx	2	—	3	(1) 98 (2) 50 (3) 10
Q70	4	1	1xxx	1	—	—	
Q327	17	11	2x, 2xx, 2xxx 5xxxx	7	—	4	(1) 95 (2) 5 (3) 99 (4) 98
Q49	20	6	3x, 2xxx, 1xxxx	3	2	1	87
Q261	4	1	1xx	1	—	—	
Q289	1	1	1xxx	—	1	—	
Q356	16	3	1x, 2xxx	3	—	—	
Q10	8	1	1xx	—	1	—	
Q72	All plates either negative or contaminated except one xxx with 10 per cent dissociated.						

X = 1 to 10 colonies per plate, xx = 10–100, xxx = 100–1,000, xxxx = more than 1,000 colonies per plate.

Fig. 1


10,240
5,120
2,560
1,280
640
320
160
80
40
20
10
TITRE
R2&3
R4&10
R7&8
R1&11
R13
R00
R9
R01
1
2
WEEKS
Rabbits inoculated with Smooth Strains
Rabbits inoculated with Dissociated Strains

RESULTS

Frequency of Occurrence of Dissociated Br. melitensis *Strains in the Milk of Goats*

The results of this part of the investigation are set out in table 1. The total number of colonies on each of the three types of medium were very similar. The proportion of dissociated to smooth colonies was also similar on the different media. Of 31 positive samples 13 contained dissociated colony types. All dissociated colonies showed typical characteristics of *Br. melitensis* except that they were negative to mono-specific melitensis serum and positive with the "dissociate" serum.

Virulence for Rabbits

The results of agglutination tests, using smooth antigens with the sera of rabbits Roo to R13 are shown in the figure. No figures are given for rabbit 12 since this was bled only once, 6 days after inoculation, and the result was negative. Rabbits 5 and 6 were negative throughout the three weeks after inoculation. There is a clear-cut difference between the response to the smooth and to the dissociated strains. R8 which received a mixture of the two types behaved as though it had been inoculated with a smooth strain.

TABLE 2

AGGLUTINATION TESTS

	Unabsorbed	
Antigen	*Abortus (99) serum*	*Melitensis (400) serum*
99 smooth abortus	640	1,280
400 smooth melitensis	1,280	2,560
423 dissociated melitensis	20	40
424 ,, ,,	20	40
409 ,, ,,	25% at 1/10	20
266 ,, ,,	25% at 1/10	40

The cultures from R12 and R13 were contaminated. All other rabbits were positive on autopsy except R9. In general the rabbits which had received smooth strains were more heavily infected than those inoculated with dissociated strains, but the differences were not spectacular at such short intervals between inoculation and autopsy. The type of strain recovered at autopsy was always indistinguishable by colony appearance after staining with crystal violet, from that injected into the rabbit R8 which received a mixture of types yielded both types on autopsy.

Agglutination Behaviour and Antigenic Structure of Dissociated Strains

Table 2 shows that sera produced after inoculation with smooth strains of *Br. abortus* and *Br. melitensis* had approximately the same titres when tested against their homologous and heterologous smooth cells. The titres of these "smooth" sera were, however, much lower when they were tested against dissociated *Br. melitensis*. Sera produced in rabbits by infection with dissociated *Br. melitensis* cells agglutinated suspensions of smooth *Br. abortus*, smooth *Br. melitensis* and dissociated *Br. melitensis* to approximately the same titre (Table 3).

TABLE 3

AGGLUTININ AND AGGLUTININ-ABSORPTION TESTS

Serum Roo	*Antigens*		
	99	*336*	*424*
Unabsorbed	320	640	640
Absorbed by 99 (abortus S) ..	—	—	320
„ „ 336 (melitensis S) ..	—	—	160
„ „ 266 (melitensis D) ..	—	—	—

Serum R13	*Antigens*		
	99	*400*	*424*
Unabsorbed	40	80	160
Absorbed by 99 (abortus S) ..	—	—	80
„ „ 400 (melitensis S) ..	—	—	160
„ „ 424 (melitensis D) ..	—	—	—

Serum from rabbit Roo, infected with dissociated melitensis strain 291.
Serum from rabbit R13, infected with dissociated melitensis strain 424.

The results of agglutinin-absorption tests performed to determine the antigenic structure of the dissociated strains are set out in Table 3. The results of agglutinin-absorption tests with these dissociated strains may be predicted by assuming their antigenic make-up to be aRm, just as results with smooth typical abortus and melitensis strains may be predicted by assuming their antigenic make-up to be Am and aM respectively, where A = abortus antigen, M = melitensis antigen and R = rough antigen, capital letters indicating major and small letters indicating minor quantities of these antigens.

Serum from rabbit Roo absorbed by strain 99 was used as "dissociate" serum in the serological typing tests mentioned earlier.

DISCUSSION

The examination of milk samples suggests a frequent occurrence of dissociated strains of *Br. melitensis* in the milk of goats in Malta. The term rough has been deliberately avoided as the crystal violet method of staining colonies does not reveal any differences in the degree of dissociation, and it is likely that many of the dissociated strains were, in fact, intermediate forms. When first isolated all the strains mentioned, including 291, which became unstable later, were stable for at least 24 hours when suspended in normal saline at 37°C. It is likely that the colonial types considered here correspond to the so-called *Br. paramelitensis* commonly mentioned in the literature of some years ago. Polding (1937) records some information regarding dissociated strains of *Br. melitensis* encountered in Malta.

These dissociated strains, while readilv infecting rabbits, produced a lesser degree of infection than smooth strains. They were markedly less active in producing agglutinins than are smooth strains. Antigens prepared from dissociated strains agglutinate poorly, sometimes hardly at all, when tested against "smooth" sera. This latter fact could easily lead to errors in diagnosis where smooth sera are used to identify brucella cultures. The results of agglutinin-absorption tests with two sera from rabbits infected with dissociated strains agree closely with those of Renoux and Mahaffey (1955) working with rough laboratory strains of *Br. melitensis* and suggest the presence of an antigen in the dissociated strain which either does not exist in the cell in the smooth phase or is masked by the presence of greater amounts of melitensis and/or abortus antigens.

It is of interest to note that strains of *Br. melitensis* isolated from goat tissues in Malta over the past two years have never shown any evidence of dissociation, though it must be admitted that no special search was made for such strains, except in one goat which we were able to autopsy at a time when it was excreting dissociated *Br. melitensis* organisms in the milk. This goat had a generalized infection and all the brucella organisms cultured from the tissues appeared to be in the smooth phase, including those from the supramammary lymph nodes. The question naturally arises as to whether the udder of the goat can act as a liquid medium and whether differences in oxygen tension between the milk and the tissues can account for the selective localization of dissociated cells in the udder.

CONCLUSIONS

The frequent occurrence of dissociated strains of *Br. melitensis* in the milk of goats in Malta is recorded.

These strains appeared to be less pathogenic and markedly less agglutinogenic for rabbits than smooth strains.

Agglutinin-absorption methods demonstrated the presence of an antigen in these dissociated strains which could not be demonstrated in smooth strains by the same methods.

Suspensions of dissociated cells were found to be somewhat resistant to agglutination by sera prepared against smooth strains either of *Br. abortus* or of *Br. melitensis*.

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FIELD TRIALS OF PROTHIDIUM AS A PROPHYLACTIC IN CATTLE TRYPANOSOMIASIS

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INTRODUCTION

Prothidium, formerly known as R.D. 2801, is one of three compounds, which combine in part the structure of Ethidium and Antrycide, produced by Messrs. Boots Pure Drug Co., and tested by Watkins and Woolfe (1956) against *T. congolense* infection in mice. Laboratory trials and toxicity tests carried out by Burdin and Fairclough (1956), showed that the drug was well tolerated as a subcutaneous injection into the dewlap in doses up to 10 mg/kg. as a 2.5 per cent solution., and that this dose protected against a needle challenge of *T. congolense* for at least 4 months.

Since large scale field trials were begun in Kenya (Anon, 1955, 1956) it has become increasingly evident that the degree of protection afforded by Antrycide Pro-salt depends greatly on the number of infected tsetse flies to which treated cattle are exposed. Longer protection is given when the number is low. Since a similar phenomenon might occur with other prophylactics, the trials described here were designed to investigate this point. They were carried out in two areas, Makindu and Ithanga; at Makindu the tsetse density was about eight times as high as at Ithanga. In order to widen the basis of the trial still more, two types of cattle and two different dosages were used. Cattle treated with Antrycide Pro-salt were also included, since this drug has been accepted as a standard of comparison for new prophylactics. In both areas untreated control groups of cattle were introduced at the beginning of and at intervals throughout the experiment.

MATERIALS AND METHODS

Definitions. For the sake of brevity a number of words have been used in the following sense. Pro-salt—injection of quinapyramine (prophylactic) B.Vet.C. Antrycide—Quinapyramine sulphate B.Vet.C. Grade—the result of the cross between exotic or European breeds of cattle and the indigenous, humped East African zebu. Positive—the appearance of *T. vivax*, *T. congolense* or *T. brucei* in either blood or gland smears of protected or control cattle. Negative—failure to find trypanosomes in either the peripheral blood or prescapular lymphatic gland juice. Challenge—exposure to bites of infected tsetse flies.

Experimental areas. Makindu comprises some 18,000 acres of typical scrub country, normally inhabited only by wild game, adjoining the main Nairobi-Mombasa road about 110 miles south of Nairobi at an altitude of 3,000 feet. The average rainfall is about 15 in. and the grazing is poor, about 20 acres per animal, and conditions are unfavourable for grade cattle. This is an area of high tsetse density. Ithanga lies about 60 miles east of Nairobi at an altitude of 4,000 feet. The grazing is comparatively good, about 9 acres to the beast. This is an area of low tsetse density.

Cattle. Both native (East African zebus) and grade cattle, were used. The zebus were bought in the Northern Frontier Province from an area believed to be free from trypanosomiasis, and were in good condition on arrival. The grade animals, which also came from areas without trypanosomiasis, varied greatly in condition and breed. All the cattle were steers between 2 and 3 years of age. No additional foodstuffs were given, but there was free access to rock salt and a mineral lick. To control tick-borne diseases, the cattle were sprayed at 3-day intervals with a proprietary brand of cattle dip containing benzene hexachloride.

Treatment. Throughout the experiment Prothidium was used as a 2 per cent solution, freshly prepared in boiling distilled water which, after cooling, was injected into the dewlap at a dosage of either 2 or 4 mg/kg. The initial treatment was given 48 hours before the cattle were railed to the experimental areas (Table 1).

TABLE 1

DETAILS OF TREATMENT OF EXPERIMENTAL CATTLE

Serial no. of group	*No. and type of cattle*	*Prophylactic treatment*	*Area*	*Tsetse density*
Makindu "A"	25 grade steers	Prothidium 2mg/kg.	Makindu	High
,, "B"	25 ,, ,,	,, 4mg/kg.	,,	,,
,, "C"	25 zebu ,,	,, 2mg/kg.	,,	,,
,, "D"	25 ,, ,,	,, 4mg/kg.	,,	,,
,, "E"	20 ,, ,,	Antrycide pro-salt 12mg/kg. (2-monthly)	,,	,,
Ithanga "A"	25 grade steers	Prothidium 2mg/kg.	Ithanga	Low
,, "B"	25 zebu ,,	,, 2mg/kg.	,,	,,
,, "C"	20 ,, ,,	Antrycide pro-salt (3-monthly)	,,	,,
,, "D"	20 ,, ,,	Antrycide sulphate	,,	,,

Prothidium reactions. No marked systemic effects were noted, although salivation and a slight uneasiness was observed in a number of cases. The irritant action of Prothidium was immediately obvious; the majority of the cattle attempted to lick or bite the site of injection. A large swelling, 10 to 20 cm. in diameter, which in a number of animals involved the whole length of the dewlap, appeared within 48 hours after inoculation and caused the majority of the cattle to walk with hesitation. The swelling, which was hot and painful when touched, gradually resolved in 7 to 14 days. In three instances abscess formation occurred which, however, readily responded to treatment. After the swelling had completely subsided marked puckering of the skin of the dewlap was noted in some cases.

Observations on cattle. Routine diagnosis of trypanosomiasis was based on the finding of trypanosomes in either the peripheral blood or in smears taken from the prescapular lymph gland. Blood slides were taken twice weekly and gland smears once a week. At Makindu, shortly after the first infections were confirmed, blood smears were examined daily. Temperatures were recorded every day.

Observations on tsetse. G. pallidipes was the predominant vector in both areas. Its density was measured as "A.D." A.D. is the number of non-teneral flies caught per 10,000 yards of patrol. Patrols were carried out twice weekly along a series of fly rounds laid out in both experimental areas. The average *G. pallidipes* A.D. at Makindu was 87 and at Ithanga, 10.5. The highest recorded A.D. to which the cattle were exposed at Makindu was 171 and at Ithanga, 22.

Intercurrent diseases. An outbreak of Foot-and-Mouth disease (Type "O") occurred ten weeks after the arrival of the cattle and resulted in deaths, lameness and loss of condition, especially among the grade animals. A second, comparatively mild, untyped outbreak was seen 10 months later. Occasional deaths were recorded from tick-borne diseases in spite of hand spraying. By the end of the first short rains, 6 months after entry, some 50 per cent of the cattle were suffering from an acute dermatitis or photosensitization believed to be of plant origin. The drug was not suspected to have caused this condition as controls and animals which had received Pro-salt were also involved. One death from Anaplasmosis and one from Blackquarter were recorded at Ithanga.

Control groups. Untreated grade and zebu cattle entered the experimental areas at the same time as the treated animals, and further groups of controls were introduced from time to time. At Makindu the first six groups of controls were left untreated in order to observe the pathogenicity of the local trypanosome; the last three groups were treated with Pro-salt as soon as infection had been confirmed. At Ithanga the first two groups of controls were left untreated and the last two received Pro-salt. Full details of control groups are given in Table 2.

TABLE 2

DETAILS OF CONTROL GROUPS

Group no.	*Month of introduction*	*No. and type of cattle*	*1st positive slide within*	*All cattle positive within*	*Average time to 1st positive*	*Average time to death positive slide seen*
Makindu F	May '56	5 grade	17 days	24 days	20 days	23
” G	”	7 zebu	21 ”	181 ”	140 ”	24
” H	July/Aug. '56	6 grade	34 ”	110 ”	50 ”	12
” I	September '56	6 zebu	62 ”	132 ”	93 ”	22
” J	October '56	4 zebu	62 ”	70 ”	67 ”	20
” K	November '56	6 grade	15 ”	29 ”	20 ”	15
” L	March '57	7 grade	11 ”	17 ”	12 ”	Treated
” M	April '57	10 zebu	12 ”	40 ”	27 ”	”
” N	May '57	5 zebu	10 ”	16 ”	13 ”	”
Ithanga E	June '56	4 zebu	48 ”	—	—	—
” F	June '56	5 grade	13 ”	25 ”	17 ”	22
” H	August '56	5 grade	16 ”	51 ”	33 ”	Treated
” G	March '57	7 grade	14 ”	95 ”	37 ”	”

RESULTS

Single Injection of Prothidium

Makindu Group A. 25 grade steers treated with Prothidium 2 mg/kg. Four animals died of extraneous causes, leaving an effective group of 21. Trypanosomes were first detected in one steer 150 days after exposure, and in two more animals 24 days later. On the 195th day trypanosomes were found in one more animal. Observations of this part of the experiment ended on the 203rd day, by which time 4/21 animals had become positive. The first three infections appeared in an average time of 163 days.

Makindu Group B. 25 grade steers treated with Prothidium 4 mg/kg. Seven animals died of extraneous causes, leaving an effective group of 18. The first positive smear was found after 227 days and the next three after 241, 251 and 255 days. During the following month nearly all the cattle showed infections and when observations ended on the 293rd day 14/18 had become positive. Two died from trypanosomiasis 16 and 35 days after trypanosomes were first seen. The first three infections appeared in an average time of 239 days.

Makindu Group C. 25 zebu steers treated with Prothidium 2 mg/kg. Three animals died of extraneous causes, leaving an effective group of 22. The first two positive blood smears were seen 209 days after exposure, and a further two 18 days later. Observations ended on the 234th day, by which time 5/22 animals were positive, The first three infections appeared in an average time of 215 days.

Makindu Group D. 25 zebu steers treated with Prothidium 4 mg/kg. One animal died of extraneous causes, leaving an effective group of 24. Trypanosomes were first seen in one steer 216 days after exposure and this animal died from trypanosomiasis 16 days later. A second animal was found to be infected on the 227th day and a third on the 248th day. When observations ended on the 297th day 13/24 had become positive. The first three infections appeared in an average time of 230 days.

Ithanga Group A. 25 Grade steers treated with Prothidium 2 mg/kg. No deaths occurred in this group from extraneous causes. Trypanosomes were first seen on the 157th day after exposure when two animals became positive. Seven days later 7/25 were infected. When observations ended on the 178th day 8/25 were positive and two of these animals died from trypanosomiasis 9 and 14 days after positive smears had first been seen. The first three infections appeared in an average time of 158 days.

Ithanga Group B. 25 zebu steers treated with Prothidium 2 mg/kg. No deaths occurred from extraneous causes. Trypanosomes were first detected after 199 days. A second animal became positive on the 245th day after exposure and a third after 252 days. Two of these animals died from trypanosomiasis 21 and 64 days after the first positive slides had been seen. The third animal recovered without treatment. Observations ended on the 322nd day when 3/25 had become positive. The first three infections appeared in an average time of 232 days.

Treatment with Antrycide Pro-salt and Antrycide Quinapyramine Sulphate

Makindu Group E. 20 zebu steers treated with Pro-salt (12 mg/kg) 2-monthly. One animal died of extraneous causes, leaving an effective group of 19. During the time between the first and second injections one animal became infected; two animals became positive between the second and third injections, and by the 189th day, when the 4th Pro-salt treatment was given, 8/19 were positive. Twenty-seven days later 10/19 were positive and the entire group was treated with Ethidium 2 mg/kg. Similar results had been obtained in previous years when cattle were grazed in this area protected by 2-monthly Pro-salt, the relapses after 6 months varying between 20 per cent and 70 per cent.

Ithanga Group C. 20 zebu steers treated with Pro-salt (12 mg/kg) 3-monthly. No deaths resulted from extraneous causes. Four treatments were given and during the 3-monthly period following each treatment no cases of trypanosomiasis occurred. Treatment ceased on the 289th day and between then and the 427th day, when observations ceased, only one animal was seen to be infected.

Ithanga Group D. 20 zebu steers treated with Antrycide (5 mg/kg) 2-monthly. Three animals died from extraneous causes leaving an effective group of 17. In the period of 427 days in which the cattle grazed in the challenge area, 8 treatments with Antrycide were given. No cases of trypanosomiasis were seen for 7 months. Then, between the 4th and 5th injection 4/19 animals became infected. Three of these responded to routine treatment with Antrycide at the end of the 2-monthly period. The 4th, however, proved resistant to both Antrycide and Ethidium (2 mg/kg) and was destroyed.

Control Groups. While the appearance of trypanosomes in blood or gland smears revealed that the cattle were no longer being protected by Prothidium, it does not allow a conclusion to be drawn as to when protection failed, for the incubation period may have been prolonged (Robson and Milne, 1957): nor is it possible to arrive at the time period of protection afforded by Prothidium by deducting an average incubation period from the first positive slide in each group, for the incubation period itself is variable and the result may be further influenced by individual idiosyncrasy or by intercurrent infection. At present, therefore, the true period of protection afforded by Prothidium is not known; it is some period shorter than the "mean negative period" quoted below.

In order to compare the results from the various Prothidium-treated groups the mean time taken for the first three animals in each group to show infection, measured from the date of exposure

TABLE 3

SUMMARY OF RESULTS AFTER ONE PROTHIDIUM INJECTION

The Antrycide protected group are shown for comparison

Group and type of cattle and treatment	*Tsetse density*	*No. positive after 4 months*	*No. positive after 5 months*	*No. positive after 5-6 mths.*	*No. positive after 7 months*	*No. positive after 8 months*	*No. positive after 9 months*	*No. positive after 12 mths.*	*Mean negative Period**	*No. positive at time when observations ceased*
Makindu A grade Prothidium 2 mg/kg.	High	0/23	1/23	3/22	—	—	—	—	163 days.	4/21 at 6½ months
Makindu B. *14* grade Prothidium 4 mg/kg.	,,	0/20	0/20	0/20	0/20	3/20	11/19	—	239 days.	14/18 at 9½ months
Makindu C. *14* zebu Prothidium 2 mg/kg.	,,	0/25	0/24	0/24	5/22	—	—	—	215 days.	5/22 at 7½ months
Makindu D. *14* zebu Prothidium 4 mg/kg.	,,	0/25	0/24*	0/24	1/24	3/24	6/24	—	230 days.	13/22 at 9½ months
Makindu E. *14* zebu Antrycide pro-salt (12 mg/kg) (3-monthly)	,,	2/19	4/19	7/19	8/19	—	—	—	—	10/19 at 7½ months
Ithanga A grade Prothidium 2 mg/kg.	Low	0/25	0/25	8/25	—	—	—	—	158 days.	8/25 at 6 months
Ithanga B. *15* zebu	,,	0/25	0/25	0/25	1/25	3/25	3/25	3/25	232 days.	3/25 at 11½ mths. No second inoculation given
Ithanga C. *15* zebu Pro-salt (3-monthly)	,,	0/20	0/20	0/20	0/20	0/20	0/20	0/20	—	1/20 at 14 months 4 Pro-salt inoculations
Ithanga D. *15* zebu Antrycide sulphate (2-monthly)	,,	0/19	0/19	0/19	0/19	4/19	1/19	1/17	—	1/17 at 14 months 7 Antrycide sulphate inoculations

* Mean time for 1st three positives to appear.

to challenge, was calculated. This is referred to as the "mean negative period". The mean of three was taken, because, at Ithanga, only three animals in one group became positive. These "mean negative periods" (Table 3) were compared statistically by analysis of variance; some comparisons were checked using the first four positives in each group. It is assumed that the true period of protection bears a relation to the "mean negative period".

In both high and low fly density areas the protection afforded by Prothidium at 2 mg/kg began to lapse about the same time, i.e. grade cattle, 163 and 158 days respectively; zebus, 215 and 232 days respectively. Increasing the dosage of Prothidium from 2 mg/kg to 4 mg/kg significantly extended the period of protection by two months in grade cattle at Makindu (2 mg/kg, 163 days; 4 mg/kg, 239 days), but no significant result was apparent in the zebu groups (2 mg/kg, 215 days; 4 mg/kg, 230 days). The difference of response is itself significant. In both high and low tsetse density areas zebu cattle, treated with prothidium 2 mg/kg, remained negative about two months longer than grade animals receiving the same dosage (215 days and 163 days respectively). Results from the Makindu area suggest that one Prothidium injection appeared to be more effective than three 2-monthly injections of Pro-salt.

Two Injections of Prothidium

All the Prothidium-treated groups except one (Ithanga Group B) received a second injection of the same drug at the same dosage and continued to graze in the challenge areas of Makindu and Ithanga as before. It should be noted that some infections had occurred in all groups before the second administration of the drug, which took place at different times in different groups (Table 4). The results of a second injection of Prothidium show a marked difference from the results of the first injection. In every group the period of protection was much shorter. This was much more obvious at Makindu, the area of high fly density, than at Ithanga.

Whereas after the first treatment at Makindu positive slides did not appear until 5 to 8 months later, after the second treatment they appeared 69 days later in Group A, 18 days later in Group B, after 35 days in Group C and 14 days in Group D. These infections were only the first of many relapses which followed in quick succession until eventually all the cattle in the Makindu groups showed infections. In Group A, 19/19 animals had relapsed by the 111th day; in Group B, 14/14 animals had relapsed by the 50th day; in Group C 22/22 had relapsed by the 77th day and in Group D there were 19/19 relapses by the 73rd day. Thus, although the groups which were given the second injection early were protected for a comparatively longer period than those groups in which the second treatment was delayed, the net result was that all groups failed about the same calendar time, regardless of when the second treatment was administered.

TABLE 4

RESULTS AFTER THE 2ND PROTHIDIUM INJECTION

Group	*No. of cattle treated*	*Day of treatment*	*% infected when treated*	*Days from treatment to 1st infection*	*Days from treatment to 100% infection*	*Mean relapse period*	*Days from start of experiment to 100% infection col. 3 – 6*
Makindu A	19	203	19	69	111	89	314
,, B	16	293	87	18	50	27	343
,, C	22	234	22	35	77	63	311
,, D	22	297	39	14	73	39	370
Ithanga A	23	178	32	32	5/25 positive in 144 days		

The failure of all the Prothidium-protected groups, coupled with the failure to cure infections in untreated controls with Prosalt, suggested that Prothidium-resistant strains of trypanosomes were being transmitted by flies and that this strain was cross-resistant to Antrycide. Pooled blood from a number of positive animals from Makindu Group C was sent in citrate to the laboratory. When this strain of trypanosomes was tested for resistance it proved to be partially resistant to Prothidium at 2 mg/kg, apparently susceptible to Berenil at 5 mg/kg, but almost completely resistant to Antrycide at 5 mg/kg and to Ethidium at 2 mg/kg. Further strains taken from untreated control cattle (Makindu Groups M and N) shortly after they were brought into the grazing area, when sub-inoculated into cattle at Kabete, were also found to be partially Prothidium-resistant and wholly resistant to Antrycide and Ethidium This result confirmed the belief that strains resistant to Prothidium were being fly-transmitted in the Makindu area.

In the area of the low tsetse density of Ithanga, Group A received the second injection of Prothidium six months after entry, by which time 8/25 had become positive although the first infections occurred only 14 days earlier. This strain was also tested at the laboratory and, although susceptible to Prothidium was resistant to both Antrycide at 5 mg/kg and Ethidium at 2 mg/kg. At this stage of the experiments no infections had appeared in any of the Antrycide-protected groups and as there had been no cattle in this area for many years there was nothing to suggest that this strain, resistant to Antrycide and Ethidium, could have come from anywhere but the cattle treated with Prothidium. The first relapse was seen in Group A (Prothidium at 2 mg/kg) 32 days after the second injection and a total of 5 occurred within 77 days. It has already been shown that a Prothidium-resistant strain, which was isolated from untreated control cattle in Makindu, was cross-resistant to Antrycide and Ethidium. Although few relapses appeared in Ithanga Group A in comparison with the Prothidium-protected groups at Makindu, and there was no evidence to suggest fly transmission at Ithanga, cross-resistance to Antrycide and Ethidium did appear and it seems probable that Prothidium resistance was just beginning to develop.

Treatment of Prothidium Relapses in the Field

When drug resistance develops during the course of a field trial, pooled blood from a number of infected cattle is sent to the Veterinary Research Laboratory at Kabete, where it is sub-inoculated into a number of cattle and tested against known trypanocides at varied dosages to determine the degree of resistance. In the meantime, the herd, in which resistance has developed, is treated with an alternative drug believed to eliminate the resistant infection and the animals moved to a fly-free area where blood slides continue to be examined daily for a period of at least three months.

TABLE 5

SPECIES OF TRYPANOSOMES IN ALL GROUPS OF CATTLE EXPOSED TO INFECTION AT MAKINDU AND ITHANGA

Group	*Place*	*Protecting drug*	T. vivax	T. congolense	*T.V/C*	T. brucei*
A,B,C,D	Makindu	Prothidium (after 1st injection)	7	14	6	10
A,B,C,D	Makindu	Prothidium (after 2nd injection)	27	148	33	—
Control Groups ..	Makindu	None	28	17	11	
E	Makindu	Pro-salt (2-monthly)	—	21	—	—
A,B	Ithanga	Prothidium (after 1st injection)	—	11	—	—
A	Ithanga	Prothidium (after 2nd injection)	—	5	—	—
Control Group ..	Ithanga	None	17	3	1	
D	Ithanga	Antrycide (2-monthly)	—	4	—	—

* *T. Brucei* was only recorded when seen as a pure infection

At Makindu, which is an area of high tsetse density, experiments using Pro-salt have been in progress for about four years. On each occasion, using Pro-salt at 12 mg/kg at two monthly intervals, *T. vivax* infections began to appear in blood and gland smears about the fifth month and, by the end of six months, between 20 per cent and 70 per cent of the cattle were infected with Antrycide-resistant *T. vivax*. At this stage the herds were treated with Ethidium at 2 mg/kg and removed to a fly-free area. On each occasion the administration of Ethidium proved effective in completely eliminating the Antrycide-resistant *T. vivax*. *T. congolense* was seldom seen in relapses and it was concluded that Pro-salt was more effective against *T. congolense* than against *T. vivax*.

During the Prothidium field trial at Makindu, shortly after 100 per cent relapses had occurred in all groups following the second injection of Prothidium, an outbreak of Foot-and-Mouth disease affected the experimental herd, and prevented its removal either to a fly-free area or for slaughter. The problem then was to find a drug or combination of drugs that would eliminate the strains resistant to Prothidium or at least keep the peripheral blood free from trypanosomes for a sufficiently long period to enable the herd to be sent for slaughter when the quarantine was eventually removed.

TABLE 6

VIRULENCE OF TRYPANOSOMES

Mean time between 1st positive blood slide and death

Makindu	V. vivax	T. congolense	T.v./T.congolense
Grade controls	13 days (7)	18 days (7)	—
Zebu controls	37 days (4)	17 days (10)	14 days (5)
Prothidium protected grades	—	21 days (7)	—
Prothidium protected Zebus	—	14 days (1)	—
Ithanga			
Grade controls	13 days (6)	13 days (1)	—
Zebu controls	48 days (2)	21 days (1)	—
Prothidium protected grades	—	28 days (3)*	—
Prothidium protected Zebus	—	42 days (2)	—

The figure in brackets ( ) denotes number of animals concerned

*One animal in this group was a "relapse" case and died after 62 days

At Ithanga, the relapses were few in number, only partially resistant, and there was no evidence to suggest that the relapse strain was being transmitted by flies.

Tables 5 and 6 summarise the available information on the species and virulence of the trypanosomes at Makindu and Ithanga.

TABLE 7

Drug	Grade Cattle			Zebu Cattle		
	No. of cattle treated	*No. positive in less than*		*No. of cattle treated*	*No. positive in less than*	
		15 days	*29 days*		*15 days*	*29 days*
Prothidium 4 mg/kg	3	0	2	2	1	0
Ethidium 2 mg/kg	8	4	4	4	2	1
Berenil	14	1	9	11	4	5
Pro-salt 16 mg/kg	5	2	2	19	0	3
Pro-salt followed by Berenil	5	0	0	5	0	0

The results of first treatments of relapses after the second injection of Prothidium at Makindu are given in Table 7. Treatment with Prothidium merely shows that the parasites were resistant to that drug. Ethidium could not be relied on to keep the peripheral blood free from typanosomes for as long as fourteen days. Of twenty-five animals injected with Berenil, at least five relapsed so quickly that they could not have been cured and re-infected again. The trypanosomes must therefore have also been resistant to this drug. As for the rest, it was impossible in the field to distinguish between relapses and re-infections. Pro-salt gave at least fourteen days of negative smears in Zebu but not in grade cattle. Quite a large percentage of the Zebu group remained negative for over four weeks. Pro-salt, followed by Berenil one or two weeks later, gave at least four weeks of negative daily smears in both grade and Zebu cattle. This combination of drugs would appear to be the most promising.

Treatment of Prothidium Relapses with Other Drugs

Eventually, all the cattle became re-infected or relapsed. They were treated again with the following results (Table 8). Berenil gave much longer protection when preceded by Pro-salt than when preceded by Berenil. Berenil as a second treament gave much the same protection as before. A second injection of Pro-salt gave much the same length of protection as the first, irrespective of whether or not it followed relapses after Ethidium or Berenil. At this stage Pro-salt, either alone or in combination with Berenil or followed by Berenil, had consistently kept trypanosomes out of the peripheral blood for at least fourteen days, and in many of the cattle for as long as two months. As soon as the Foot-and-Mouth quarantine was removed the whole experimental herd was treated with Pro-salt and Berenil, given simultaneously, and moved to an area of low tsetse density. During the following sixty days infections were seen in 5/65 animals. Pro-salt and Berenil was again administered but this time an interval of seven days was left between the injections. When the herd was sent for slaughter seven weeks later all the cattle were still negative.

Treatment of Prothidium Relapses in the Low Tsetse Density Area

After the second Prothidium injection 5/23 relapses occurred within seventy-seven days. One animal was left untreated and died fifty-nine days later. The remaining four animals were treated with the following results: Berenil, at 5 mg/kg, cured 2/2; Ethidium, at 2 mg/kg, cured 1/2.

The strains of *T. vivax* and *T. congolense* collected from the first infections before the second injection of Prothidium was given, when tested at the Laboratory were found to be resistant to Antrycide and Ethidium but not to Prothidium or Berenil. Because Ethidium at 2 mg/kg had been so effective in eliminating the Makindu strain of Antrycide-resistant *T. vivax*, it was the first drug

TABLE 8

RESULTS OF TREATING CATTLE WHICH HAD RELAPSED AFTER PROTHIDIUM

The figures are the negative period in days which followed after treatment with other drugs

Group	*P*	*Ethidium*		*Berenil*				*Antrycide*			*A/E*			*A/B*			*A+B*		*B+B*		*A+A*	*A+A +B*
	1	*1*	*3*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*2*	*3*	*4*	*2*	*3*	*4*	*1*	*2*	*1*	*2*	*2*	*1*
Makindu A. Grade cattle	18 32 25	18 17 14 17 4 14 14 15	17	25 7 18 22 19 22 34 26	13 16 11 24 36 66 31 8 34 20	36 26	11*		41 43 48 27 11 13	39 48 56 35 35* 35 25		21 26 47* 25	14 53 33* 29* 36* 19*		28	75* 37 77*		75 53 68				
Makindu B grade cattle		9†		17 17 35 29 25 41	26	21		23 10 6 15 62	8 68 43 12 19	39*	54* 56* 47*	54* 33 28* 47* 33*	45	11	31 55*	45	51* 52*		25	47 49		

TABLE 8—*Continued*

Group	*P*	*Ethidium*		*Berenil*				*Antrycide*			*A/E*			*A/B*			*A+B*	*B+B*		*A+A*	*A+A +B*
	1	*1*	*3*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*2*	*3*	*4*	*2*	*3*	*4*	*1*	*1*	*2*	*2*	*1*
Makindu C. Zebus	55 8	14 51 19 7	4	18 6 16 30 34 5 19 18 9 11 16	70	68 109*		18 60	109* 53 64 65 100 76 68 87 38 17 63 65 79* 58*	23 13		58 28* 29*			19* 81* 19*		139* 74 139* 86†			58	117*
Ma'kindu D Zebus								100 46 69 85* 37 99* 50 71 62 93 81* 21 106* 47 46 83 27		27* 47* 29* 51 28*				63* 19* 81*			76 109*				

1 = First treatment
2 = Second treatment
3 = Third treatment
4 = Fourth treatment

* = Still negative when experiment closed
† = Second Treatment
P = Prothidium 4 mg/kg.
E = Ethidium 2 mg/kg.

B = Berenil 5 mg/kg.
A = Antrycide pro-salt 16 mg/kg.

used when relapses occurred in the Prothidium field trials at Makindu.

Ethidium proved unsuccessful, and the reason is obvious. Since Prothidium contains a phenanthridine group, trypanosomes resistant to this drug may be expected to show resistance to other drugs containing this group, of which Ethidium is one. Prothidium also contains a pyrimidine group which is one constituent of Antrycide. A partial cross-resistance to Antrycide might therefore have been expected. The field results suggest that cross-resistance to Antrycide did exist, since a number of animals again became positive within a comparatively short time after treatment with Pro-salt. The drug Berenil (a diamidine) is chemically unrelated to either Antrycide or Prothidium. It was, therefore, surprising to find that this did not invariably effect a cure in the field. The only possible conclusion is that trypanosomes resistant to Prothidium are partially cross-resistant to Berenil as well. After investigation, this fact was confirmed in the laboratory.

DISCUSSION

Robson and Milne (1957) demonstrated that where a breakdown to Prothidium did occur, the period between needle challenge and the appearance of trypanosomes in the blood was considerably increased. In some cases the incubation period was extended to 50 days. It is possible that, when the Makindu groups were treated with Prothidium for the second time, instead of only a percentage of the group being infected, as judged by the evidence of blood and gland smears, all the cattle were suffering from suppressed infections and the second injection only served to prolong the incubation period.

The Makindu experiment, because of the high tsetse density and the length of time before some of the groups received a second treatment, represented a severe challenge to Prothidium prophylaxis. Ithanga, on the other hand, was an area where cattle were shown to have been adequately protected by Antrycide (2-monthly). In the case of Ithanga Group A, although Prothidium was administered within 14 days of the appearance of the first infections, relapses occurred and there was evidence of incipient Prothidium resistance. This result in particular suggests that Prothidium prophylaxis begins to fail at some period—possibly as long as 50 days—before positive blood smears are seen. In order, therefore, to avoid the onset of resistance this danger period must be taken into consideration, and it is suggested that the cattle should be treated at intervals of 3 and 4 months, depending on whether they are grade or Zebu cattle.

Once resistance to Prothidium appears in an area it may prove difficult to eliminate. If it is only in the early stages, Berenil is likely to be the most successful of the curative drugs in common use, provided the cattle can be removed to a fly-free area. Once trypanosomes resistant to Prothidium are well established in the cattle, however, no single drug can be guaranteed to effect a cure.

At Makindu, the combination of Pro-salt at 12 mg/kg, followed by Berenil at 5 mg/kg seven days later seems to have suppressed and may even have cured the infections.

Although Prothidium is potentially an excellent prophylactic, capable of protecting cattle for a period of 5 months after one injection even in areas of high tsetse density, the readiness with which resistant strains appear to emerge after one treatment, and the lack, as yet, of another drug capable of eliminating these strains, would appear to indicate the desirability of further field trials before the drug is put into general use as a prophylactic in the field.

It has recently been demonstrated at the Veterinary Research Laboratory, Kabete, Kenya, that a Prothidium-resistant strain can be eliminated by one intramuscular injection of Metamidium (May & Baker) at 4 mg/kg. (Whiteside, personal communication).

CONCLUSIONS

A series of experiments are described in which Zebu and grade cattle have been grazed in areas of high and low tsetse densities protected by Prothidium.

After a single injection at dosages of 2 mg/kg or 4 mg/kg blood smears from the cattle remained negative for periods of 5 to 8 months.

A second injection of Prothidium was followed by the appearance of drug resistance in both areas and the attempt to eliminate the resistant strains of trypanosomes by the use of other drugs is described.

By the use of Pro-salt, followed by Berenil seven days later, it was possible to keep the peripheral blood free from trypanosomes until the cattle were sent for slaughter.

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THE REACTIVITY OF THE RUMINANT UTERUS TO POSTERIOR PITUITARY HORMONES

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INTRODUCTION

The reactivity of the myometrium to extracts of the posterior pituitary gland is known to vary with the state of sexual activity. Much of the work in this field has been in laboratory animals and the clearest relationships have been established in the rabbit. Knaus (1926) reported that pregnancy in this species is accompanied by decreased response of uterine strips to pituitrin, but that at parturition the muscle shows high reactivity. He further showed that depression of uterine activity is associated with the activity of the corpus luteum (1930, 1934) and his views found early confirmation (Siegmund, 1931; Illingworth and Robson, 1932; Robson, 1933, 1935, 1936; Makepeace, Corner and Allen, 1936). In contrast to the low sensitivity produced by corpus luteum extracts and progesterone, it was shown that treatment with oestrogens augments reactivity to posterior pituitary hormones (Reynolds, 1930; Morgan, 1933, Robson, 1933, 1936). Recent work at the Carnegie Institute has done much to clarify the mechanism of the depressant action of progesterone and the augmentor effect of oestrogen in terms of simple biophysical changes and has more clearly associated progesterone dominance with the depressed uterine reactivity of pregnancy, and oestrogen dominance with high reactivity at parturition (Csapo, 1956; Schofield, 1957).

The well defined changes in uterine responses to posterior pituitary hormones seen in the rabbit have not been so unequivocally demonstrable in other species, but general points of similarity have been recorded repeatedly in man and in mice, rats, guinea-pigs and cats. The evidence is well reviewed by Reynolds (1949) and Marshall and Moir (1952).

In ruminants, although it has been shown that uterine muscle responds to posterior pituitary fractions in vitro (Newton, 1934; Alexander, 1945; Asdell, de Alba and Roberts, 1945) and in vivo (Van der Kaay, 1928; Benesch and Steinmetzer, 1931), almost nothing is known of the variation of this response in relation to sex hormone secretion. Evans and Miller (1936) have briefly described, but without published objective evidence, a variation in uterine reactivity to pituitrin during the ovarian cycle in normal intact cows. Jordan (1952) described an apparent increase in uterine response to posterior pituitary extract seen in two puerperal cows 30 minutes after the intravenous administration of stilboestrol dipropionate, but these observations were made incidentally and were not fully controlled.

Most of the published reports, including all those concerning ruminants refer to the use of unfractionated posterior pituitary extracts, which contain both vasopressin and oxytocin. Although oxytocin is the principal uterine stimulating hormone it has been shown that vasopressin may elicit contraction of the uterus in rabbits, rats and guinea-pigs (Bachinsky and Allmark, 1947; Stewart, 1949) and man (McLellen, 1940; Gardiner and Bradbury, 1940; Moir, 1944; Schild, Fitzpatrick and Nixon, 1951). No published work has been seen of the separate effects of the two fractions on the ruminant uterus.

Experiments were undertaken to study uterine reponse to posterior pituitary preparations in the cow, including variations in reactivity associated with the action of sex hormones. The results have application to the use of posterior pituitary extracts in parturient ruminants.

METHODS

Intrauterine pressure was recorded from small (2 ml.) ballons of thin latex rubber communicating by water transmission with recording mercury manometers, as previously described in detail (Fitzpatrick, 1957). All responses to pituitary hormones are expressed as the maximum rise in intrauterine pressure in millimetres of mercury. In non-pregnant cows the balloon was situated at the level of the bifurcation of the uterine horns and in the pregnant cow and sheep it was in contact with foetal membranes or foetal fluid.

Most of the pregnant cows were used on more than one occasion, without producing abortion. Catheterisation was effected per vaginam and for this procedure and for all recordings in cows, posterior epidural anaesthesia was maintained with procaine hydrochloride. In sheep, laparotomy was necessary to facilitate catheterisation per vaginam, and for this and the subsequent observations, general anaesthesia was maintained with cyclopropane or sodium pentobarbitone. In both cows and sheep after inserting the balloons a period of 45 minutes elapsed before any drugs were given.

The preparations of posterior pituitary hormones used were "Pitocin", "Pitressin" and occasionally "Pituitrin" of Parke, Davis and Company Ltd. "Pitocin" is standardised to contain 10 oxytocic units, and not more than 0.5 pressor unit, per ml. "Pitressin" is standardised to contain 20 pressor units, and not more than 1.0 oxytocic unit per ml. "Pituitrin" is standardised to contain 10 oxytocic units, and 8 to 10 pressor units per ml. Throughout this report doses will be quoted entirely in International Units and where comparison of potency is made between "Pitocin" and "Pitressin" it will be on a basis of unitage and not volume of solution.

All injections of pituitary hormone solutions were administered, unless otherwise specified, through an indwelling venous cannula of polythene, as previously described (Fitzpatrick, 1957). Some animals were treated with stilboestrol which was given as the dipropionate in oil by intramuscular injection.

RESULTS

Preliminary Experiments

Tachyphylaxia. It has been known for some years that uterine muscle may show tachyphylaxis to repeated doses of pituitrin. This has been attributed by some authors to the vasopressin fraction rather than the oxytocic fraction whilst others claim that tachyphylaxis to oxytocin may also occur.

Separate observations by the author (Fitzpatrick, 1955) on the human uterus indicated that diminution of response to repeated administration of a constant dose of oxytocin was insignificant, but that when a dose of vasopressin was interposed between successive equal doses of oxytocin the second oxytocic response was significantly reduced ($P = 0.05$). The nature of these recordings indicated that after vasopressin administration a period of inhibition persisted for approximately 20 minutes during which the myometrium was relatively refractory to further hormonal stimulation. This phase was observed to end abruptly, suggesting that this period of decreased reactivity was due to vasoconstriction within the uterus which interfered with the subsequent action of oxytocin by denying it access to uterine muscle cells. This is in agreement with the work of Weinstein and Friedmann (1935) who showed that in the rabbit the time interval between injections was the most critical factor in determining the incidence of tachyphylaxia after vasopressin. Cross (1958a) has recently reported similar findings in the rabbit.

A preliminary series of 8 experiments in cows was undertaken to test whether tachyphylaxia occurred under the conditions of the experiments to be described in this paper. With repetition of the dose of oxytocin at intervals of between 20 and 30 minutes only slight diminution of response was detected which was not significant according to the "t" test. Responses to oxytocin given 20 to 30 minutes after vasopressin were consistently less than responses to the same dose of oxytocin given prior to vasopressin. The mean diminution in response to oxytocin under these conditions was 22 per cent., but this effect of vasopressin was very variable and no statistical significance can be attributed to it at the probability of 0.05.

Finally, we found that repeated administration of vasopressin in large doses (30 units) and of Pituitrin (35 units) gave no evidence of tachyphylaxia when an interval of 40 minutes between injections was adhered to. This period was taken as a standard interval between injections in all the experiments to be described.

Relative potency of oxytocin given intravenously and intramuscularly. Responses to oxytocin were compared after intravenous and intramuscular injection in two preliminary experiments in non-pregnant cows. As expected, the responses after intravenous injection were the greater, the relative potencies being 2.5 and 2.25 in the respective experiments, one of which is illustrated in Fig. 1. Whereas the responses after intravenous injection commenced dramatically within 30 seconds and reached maximum intensity immediately, those after intramuscular injection were not apparent for some 3 minutes and took a further five minutes to reach a maximum.

Fig. 1

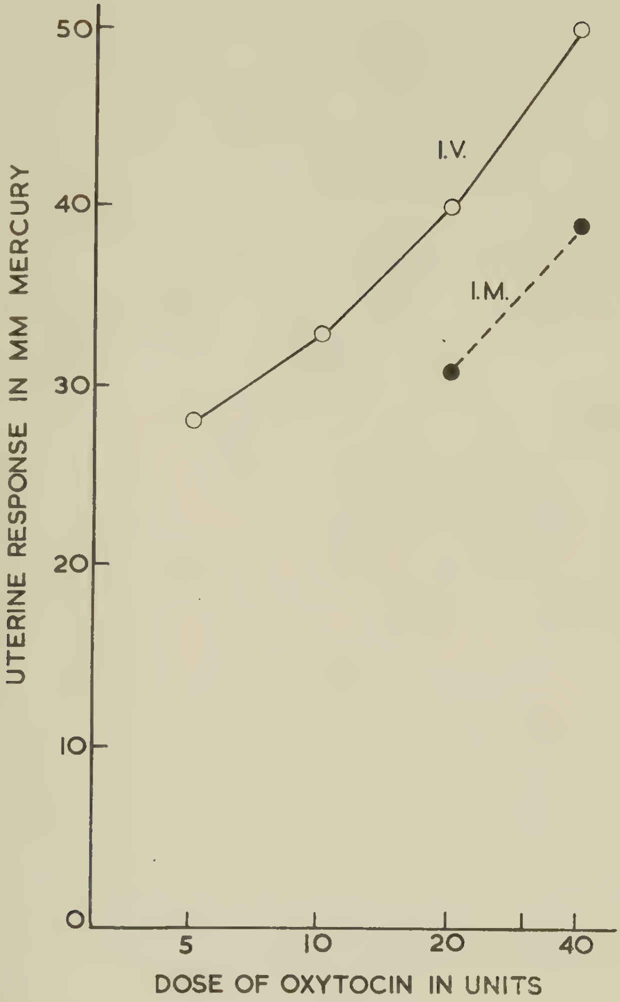

Fig. 1. Non-pregnant cow, single experiment. Increase in intrauterine pressure is plotted against logarithm of dose of oxytocin.

Responses after intravenous administration (I/V) show satisfactory regression against dose. Equal magnitude of response after intramuscular injection (I/M) is obtained when the dose is increased approximately 2½ times.

With the doses used, up to 60 units, responses were over within 25 or 30 minutes and no difference was detectable between the two routes of administration in this respect. This will be further discussed at the end of this paper.

General Results

Responses to oxytocin in cows. Ten cows were examined repeatedly at varying stages of pregnancy, close to parturition and when non-pregnant. The latter results will be presented first.

None of the recordings in non-pregnant cows quoted below were made at natural oestrus or pro-oestrus. The majority were made in the luteal stage of normal ovarian cycles, but in three

TABLE 1

OXYTOCIN INDUCED RESPONSES OF NON-PREGNANT BOVINE UTERUS
Expressed in m.m. mercury.

Animal	*Dose of oxytocin in units i/v.*										
	½	1	2	3	4	5	6	8	10	30	60
Untreated Cows											
16 a		3	4				15				
16 b						11½					
99										42	52½
23 a						16					
23 b				8							
23 c		5		9½				15			
23 d		4½					6				
25						12½					
2 a			7½		15			18			
2 b			5		10						
68 a	2½		7½								
68 b				9							
68 c			7½					15			
68 d	4½	8									
24			5								
22 a			4		7			17½	24		
22 b						12					
22 c									15		
*Treated Cows**							*Dose of Stilboestrol*		*Duration of action*		
22	14			25			30 mg.		3 days		
23	20	27½					20 mg.		2 days		
16 a		5					50 mg.		1 day		
16 a		20				35			3 days		
16 a		28							7 days		
24			29	32			35 mg.		3 days		
16 b			22				20 mg.		3 days		

In the first column the suffixes indicate completely separate experiments in the same individual animal.

* The cows receiving stilboestrol were initially in anoestrus of long duration.

experiments the animals were in anoestrous of long duration. The responses over the whole group of non-pregnant cows were surprisingly regular as indicated in Table 1. There is a satisfactory regression between dose and effect, and when expressed graphically as in Fig. 2 the responses appear to lie on the bottom half of an S—shaped log.dose/ response curve. Although responses were not studied at natural oestrus, some of these animals were used after the intramuscular administration of from 20 to 50 mg. stilboestrol dipropionate in oil as reported in the bottom half of Table 1. In all cases oestrogen temporarily augmented the response to oxytocin as illustrated in Fig. 2 and over the range tested the potency of oxytocin increased approximately tenfold. A similar augmentation was seen at parturition.

Fig. 2

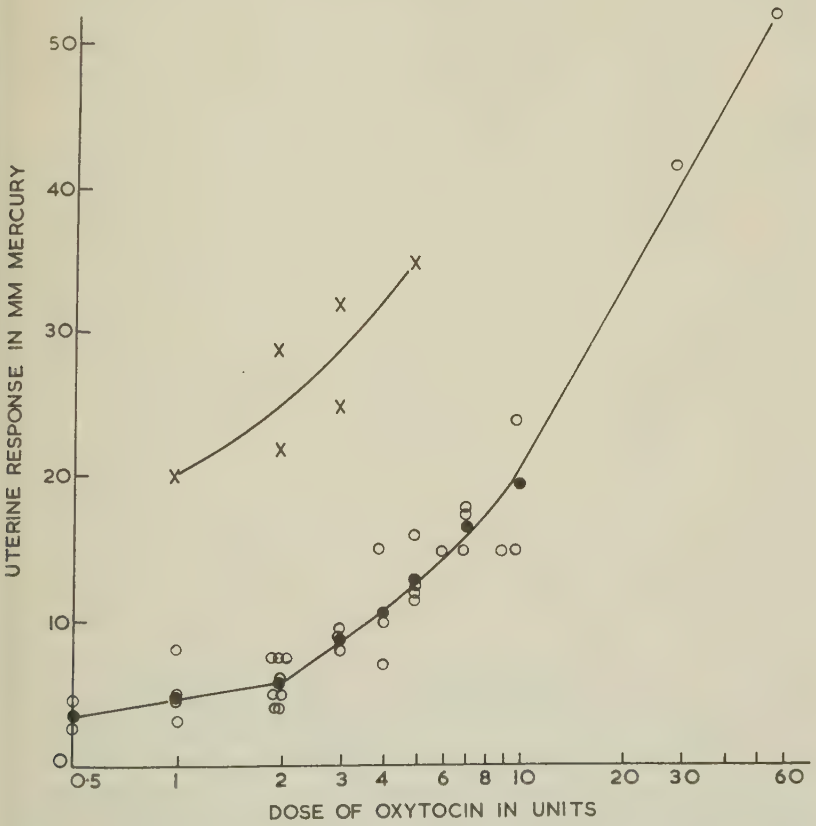

Fig. 2. All uterine responses to oxytocin obtained in non-pregnant non-oestrous cows are shown as a log-dose response curve. Open circles indicate individual observations and closed circles indicate the mean response at each dose. Also shown are responses obtained on the third day after intramuscular injection of stilboestrol (crosses).

Using 6 cows, 4 of which were also studied when non-pregnant, twelve experiments were made near the middle of pregnancy and nine near term or within 48 hours of delivery. The results obtained are presented in Table 2 and indicate that the uterus is more sensitive to oxytocin towards the end of pregnancy than between the 20th and 30th week. Thus at the end of pregnancy pronounced responses were recorded after 1 unit oxytocin or less whereas such doses at mid-pregnancy produced at the most very feeble responses. Further, in the only animal available to us at a relatively early stage of pregnancy (11 weeks) a very low reactivity was recorded. This change in reactivity during pregnancy is illustrated in Fig. 3 and is especially rapid and short-lived close to parturition. This was particularly obvious in two animals which were observed repeatedly about this time. Thus in cow 16 consistently moderate

TABLE 2

THE RESPONSE OF THE PREGNANT BOVINE UTERUS TO OXYTOCIN

Responses expressed in m.m. of mercury.

Duration of pregnancy	*Cow*	*Dose of oxytocin in Units i/v.*								
		¼	½	*1*	*2*	*3*	*4*	*5*	*7½*	*20*
Mid-pregnancy										
11 weeks	20									2
19 :	20								10	
21 :	18			2½	4			8		
21 :	68		0	3		4½				
22 :	20		0	0	6				16	
24 :	68			5½		11½				
25 :	20		2		11					
26 :	22						14			
27 :	22	0	0	1½	5				18½	
28 :	20				2½		7			
30 :	22					4½		12		
30 :	18		0	2					19	
Late pregnancy										
Term–14 days	16			9	12					
: – 7 :	16			9	11	14				
: – 5 :	23				12		18			
: – 3 :	16			7	11	13			18	
: – 1 :	16			10	15		20			
: – 1 :	23	7	14	33						
Post-partum										
24 hrs.	23			4			8			
40 hrs.	16				27½		41			
48 hrs.	68		33	52						

responses were obtained at 4 examinations over the last two weeks of pregnancy and even up to 24 hours before delivery, yet 40 hours postpartum reactivity was very much higher. Similarly in cow 23 reactivity was highest when examined the day before delivery—some six times higher than when examined four days earlier and ten times more reactive than when examined 24 hours part-partum. Cow 68, which unfortunately could not be examined repeatedly about the time of parturition, also showed very high reactivity the highest in the series, 48 hours before delivery. It should be

Fig. 3

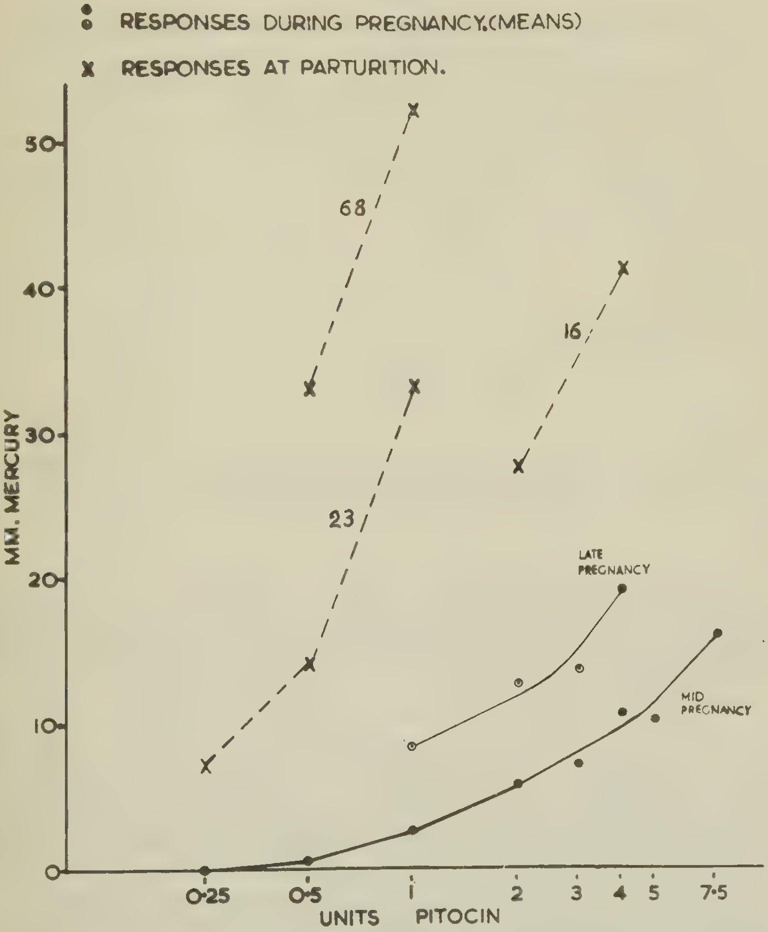

Fig. 3. Magnitude of response is plotted against dose of oxytocin for cows at mid-pregnancy (19 to 30 weeks), late pregnancy (38 to 40 weeks) and at parturition. The points for mid-pregnancy and late pregnancy are the means of several animals. At parturition the relationship is shown separately for individual animals (broken lines).

noted that at the height of reactivity the small doses used elicited responses of between 30 and 50 mm mercury, i.e. of a magnitude recognised as adequate for the progress of normal parturition in man (Caldeyro-Barcia and Poseiro, 1958).

Relative activity of vasopressin and oxytocin in cows. Vasopressin was administered to pregnant and non-pregnant cows following much the same plan as with oxytocin. In many experiments both oxytocin and vasopressin were given in such a manner that their potencies could be compared.

TABLE 3

THE RESPONSE OF THE NON-PREGNANT BOVINE UTERUS TO VASOPRESSIN

Responses expressed in m.m. of mercury

Cow	*Dose of vasopressin in units i.v.*									
	5	*6*	*9*	*10*	*14*	*15*	*18*	*20*	*30*	*40*
16 a)			7½							
b)	2					10				20
c)	3									
23 a)									9	
b)				5				7½		
22 a)				10			13			
b)		5½								
68 a)		1½			4					
b)			3½			5½				
2								12		16

In the first column the suffixes indicate completely separate experiments in the same individual animal.

As with oxytocin, a satisfactory regression between dose and effect was obtained for vasopressin in non-pregnant, non-oestrous cows as illustrated in Figs. 4 and 5 and Table 3. In five of these experiments multiple injections of both oxytocin and vasopressin were given and from the resulting concentration action curves, which do not deviate significantly from parallelism ($P=0.05$), it was possible to express the relative activity of the two hormones, somewhat as described by Schild (1942). The five estimates of the relative potencies so derived are given in Table 4 and, although showing considerable variation, oxytocin was in all cases more potent than vasopressin, the geometric mean of the ratio of activities

Fig. 4

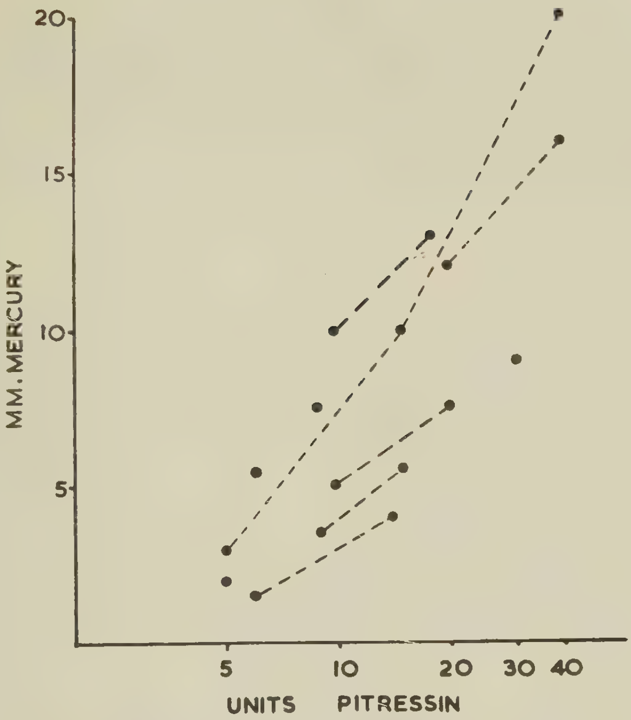

Fig. 4. Magnitude of response of the non-pregnant, non-oestrous bovine uterus is plotted against dose of vasopressin. Observations from the same experiment are joined by broken lines; these lines have similar slopes.

being 5·9. These figures bear upon the problem of whether the activity of the vasopressin solution used was due to the vasopressor polypeptide or to the inevitable contamination during extraction with up to 5 units per cent of the oxytocic polypeptide. The results (Table 4) when interpreted by the "t" test indicate that the activity of our solution of vasopressin is very significantly greater ($P = 0.01$) than that attributable to such contamination. This is further discussed below.

In three experiments in which stilboestrol dipropionate was administered to cows it was possible to study responses to vasopressin as well as to oxytocin. In all cases a temporary augmentation of reactivity occurred as with oxytocin. The results are presented in Table 5. The augmentation of response associated with the oestrogen is approximately fourfold, and is considerably less than that for oxytocin (See Table 1) despite the fact that the observations were made during the same experiments. Such a difference of augmentation implies a change in the relative activity of the

Fig. 5

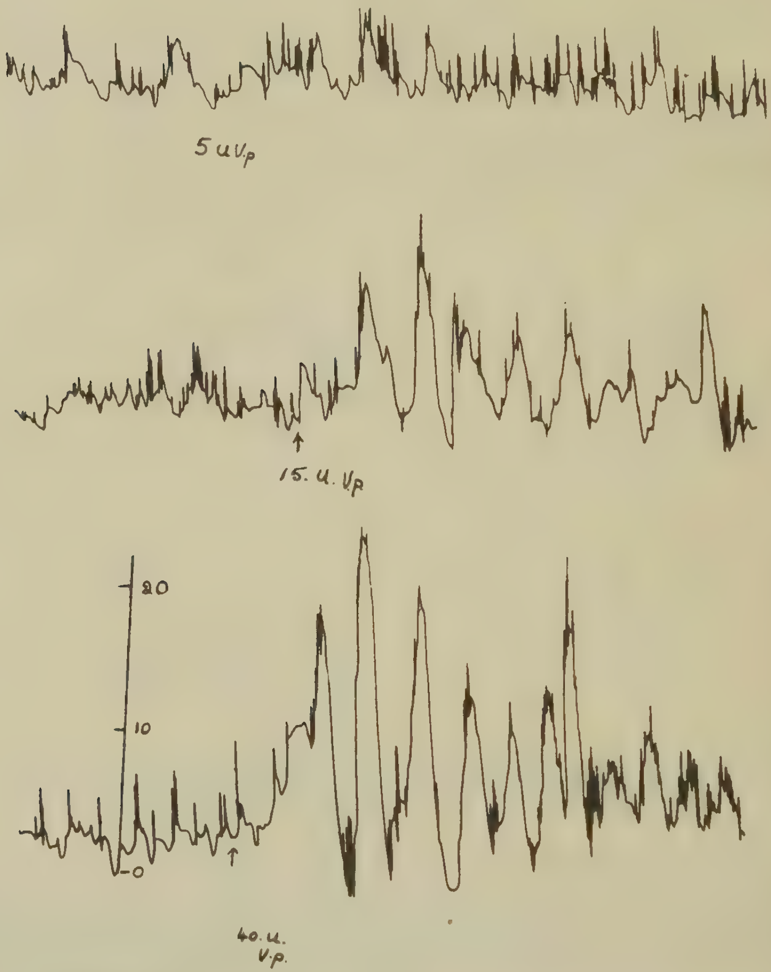

Fig. 5. Kymograph tracings of the responses of the uterus of a non-pregnant cow given increasing doses of vasopressin intravenously. The magnitude of response is proportional to dose.

two drugs and this is reflected in the three individual experiments described in which the ratios of potency of oxytocin to vasopressin were 4.0, 4.0 and 2.5 before oestrogen and 5.5, 7.0 and 10.0 with oestrogen. These results in intact cows are keeping with the work of Stewart (1949) who found that the uterus of stillboestrol-treated guinea-pigs given stilboestrol is more sensitive in vitro to both vasopressin and oxytocin than that of animals given progesterone, but that the increase in sensitivity is greater for oxytocin than for vasopressin.

TABLE 4

RELATIVE ACTIVITY OF OXYTOCIN AND VASOPRESSIN IN NON-PREGNANT COWS

Cow	*Doses (in Units) producing equivalent effects*		*Relative potency*
	Oxytocin	*Vasopressin*	*Vasopressin : Oxytocin*
22	5.3	13.5	1 : 2.5
16	3.5	13.25	1 : 3.8
2	4.15	28.0	1 : 6.75
23	1.65	11.5	1 : 7.0
68	0.86	11.7	1 : 13.6

Responses to vasopressin were studied in pregnant cows and in cows close to parturition. In all experiments the animals were also treated with oxytocin so that it was again possible to estimate the relative potency of the two preparations. The results are presented in Table 6 and show a general similarity to those found for oxytocin, with a temporary increase in reactivity close to par-

TABLE 5

THE RESPONSE OF THE BOVINE UTERUS TO VASOPRESSIN AFTER ADMINISTRATION OF OESTROGEN

Responses expressed in m.m. of mercury

Cow	*Dose of Stilboestrol dipropionate*	*Duration of action*	*Dose of vasopressin in units*							
			1	*3*	*5*	*6*	*9*	*15*	*30*	*40*
16a	0	Before			3		7½			
	50 mg.	3 days			9					
		5 days						27		
16b	0	Before			2			10		20
	20 mg.	3 days		9			16½	23	35	
22	0	Before				5½				
	30 mg.	2 days	3		11					

Experiments (a) and (b) using Cow 16 were separated by an interval of three months.

Each animal was initially in anoestrus of long duration.

TABLE 6

THE RESPONSE OF THE PREGNANT BOVINE UTERUS TO VASOPRESSIN

Responses expressed in m.m. of mercury

Duration of pregnancy	*Cow*	*Dose of vasopressin in units i/v*							
		2	*3*	*4*	*6*	*8*	*10*	*18*	*20*
Mid-pregnancy									
19 weeks	20		0			0		½	
21 :	18						2½		
21 :	68		0		0	0			5
22 :	20			0			0		0
24 :	68		0			6			
25 :	20	0			3½				13½
26 :	22						3		8
27 :	22			0			0		14
30 :	18		3		4½				
Late pregnancy									
Term—14 days	16					6	13		
: —7 :	16	5		11		12½	14		
: —3 :	16					14		20	
: —1 :	16					13			
: —1 :	23					15			
Post partum									
24 hrs.	23			8			11		15
40 hrs.	16					35			
48 hrs.	68					37			

turition. Some differences can be detected however between the responses to the two drugs. The reactivity to vasopressin appears to increase from the 38th to 40th week whilst that to oxytocin remains constant (Fig. 6). On the other hand the dramatic increase in response to oxytocin seen at parturition is not seen with vasopressin. After delivery reactivity to oxytocin falls to a low level, before that to vasopressin. These differences are strikingly illustrated by estimating the activity ratio of oxytocin to vasopressin in individual experiments. The estimates so obtained are plotted in Fig. 7 against duration of pregnancy. Up to the 38th week of pregnancy the ratio remains reasonably constant between 10.0 and 7.5, but in the last week of pregnancy a sudden fall towards equipotency occurs, coincident with the increased activity to vasopressin. Immediately prior to parturition this trend is reversed coincident with the markedly increased reactivity to oxytocin and the ratio proceeds to a maximum. Within 24 hours of delivery however, the ratio has fallen to low values indicating that the state

Fig. 6

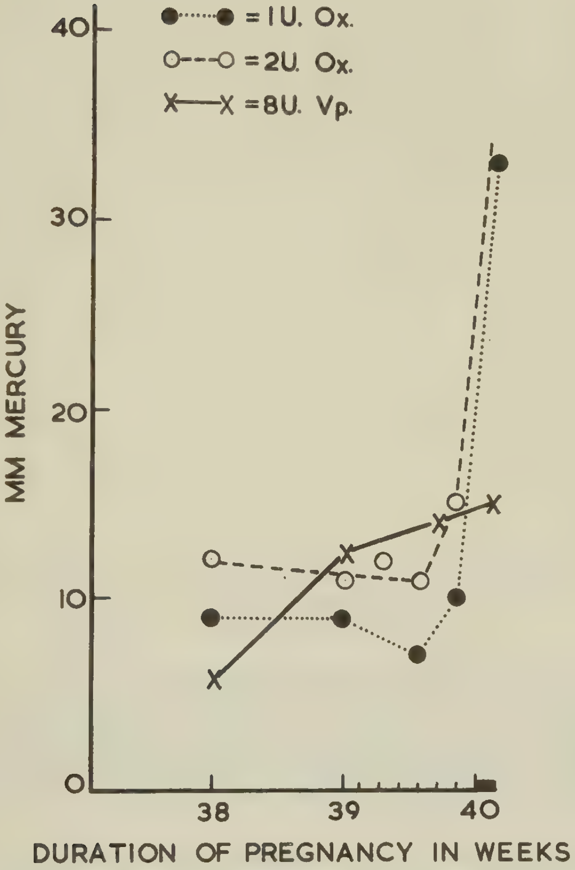

Fig. 6. Uterine responses to oxytocin (OX) and vasopressin (Vp) are compared during the last two weeks before delivery. Reactivity to vasopressin appears to increase consistently over the period whereas that to oxytocin does not increase until parturition is imminent.

of high reactivity has already passed for oxytocin, but not for vasopressin.

Reactivity of the uterus to oxytocin and vasopressin in pregnant sheep. Observations similar to those in pregnant cows were made in thirteen pregnant sheep and in five sheep shortly after lambing. Each ewe was used once only during pregnancy. The recording technique was similar in principle to that used in bovines, but in all cases the sheep were anaesthetized with either sodium pentobarbitone or cyclopropane. The duration of pregnancy in the sheep available for study, as estimated from the size of the foetuses (Barcroft, 1946), ranged from 11 to 21 weeks. Observations were

Fig. 7

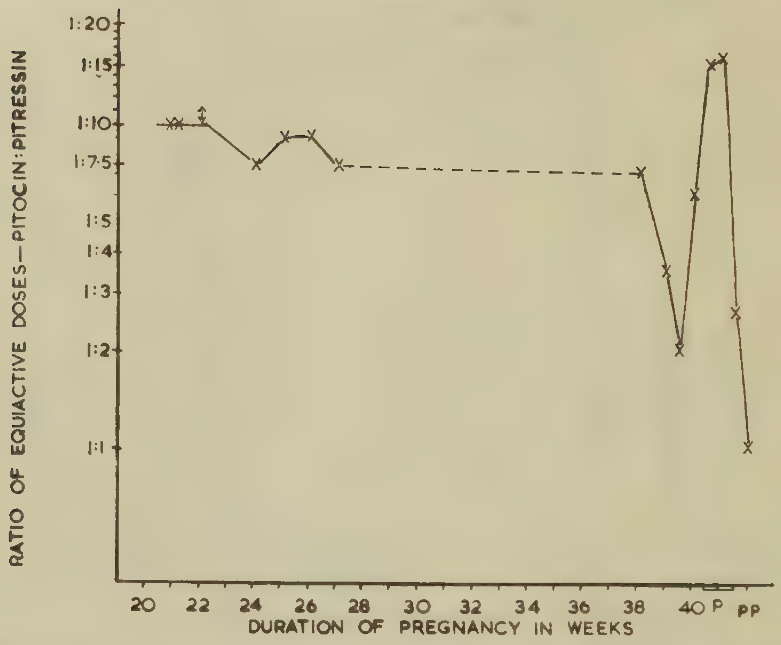

Fig. 7. The relative potency of oxytocin ("Pitocin") and vasopressin ("Pitressin") changes as pregnancy advances. In mid-pregnancy oxytocin is approximately seven to ten times as potent as vasopressin, but during the last two weeks of pregnancy vasopressin is more active. At parturition this trend reverses and oxytocin is much more potent than hitherto. Immediately after parturition reactivity to oxytocin is lost more quickly than to vasopressin so that the ratio of activities again approaches unity.

thus restricted to the second half of pregnancy, but within this period were more evenly distributed than with the cows.

There was excellent regression between dose of oxytocin and magnitude of uterine contractile response (Table 7). Reactivity increased relatively little between the thirteenth and nineteenth weeks of pregnancy, but at parturition there was a dramatic increase similar to that observed in cows. After delivery this high reactivity was rapidly lost. It was not possible to examine the same ewe repeatedly as was done in cows; nevertheless the results clearly indicate that exceptionally strong responses, very much greater than those of pregnancy, occur close to parturition. The suddenness of the increased response can be seen by comparing the high responses in sheep No. 40 at full term but some hours before labour became evident, with the moderate responses observed consistently in other sheep over the last few weeks of pregnancy, including sheep No. 4 which was studied on the expected date of parturition but in fact 3 days before delivery occurred. The short lived nature

TABLE 7

RESPONSE TO OXYTOCIN IN PREGNANT AND PUERPERAL SHEEP

Responses expressed in m.m. of mercury

Sheep	*Duration of pregnancy*	*Dose of oxytocin (i/v)*					
		250 mU	*500 mU*	*1 U.*	*2 U.*	*4 U.*	*5 U.*
100	11 weeks		3		4½		9½
39	13 :			4			
7	14 :			6			
5	15 :			6½		17	
9	16 :	2	3½				
99	16 :				10½		
3	17½ :			8	10½	13½	
6	18 :		5½				
97	18½ :	2			10		24
2	19 :		4½				
37	20 :			9		22	26
4	21 :		5	10½			
40	Parturition			24			
38	P+12 hrs.	7½	11½		33		
98	P+ 2 days	2	3½				
38	P+ 3 days		2	9		14	
37	P+ 4 days		0	7½			
1	P+ 5 days	0	2	6	7½		

of this augmentation is seen by comparing the high response obtained less than 12 hours after delivery in sheep No. 38 with the weak responses in this and other sheep on the second to fifth days post partum.

The dose most consistently administered was 0.5 unit and the responses to this dose between the second and fifth day of the puerperium were significantly less (at $P=0.02$) than the responses to the same dose given in pregnancy, excluding parturition. This agrees with the observation of very low reactivity to oxytocin soon after parturition in the cow.

In ten pregnant and five puerperal ewes, vasopressin was administered as well as oxytocin. The doses ranged from 2 to 30 units given intravenously and although on many occasions only feeble responses were obtained, the dosage could not be increased further owing to the dangerous changes in circulatory function produced by the pressor polypeptide. Clinically detectable effects were produced with five units in all ewes. Two animals died from cardiac embarrassment immediately after the administration of only 4 units vasopressin intravenously. Table 8 indicates that between the 11th and 16th week of pregnancy the responses were feeble, but between the 17th week and full term the responses increased. At

TABLE 8

RESPONSE TO VASOPRESSIN IN PREGNANT AND PUERPERAL SHEEP

Responses expressed in m.m. of mercury

Sheep	*Duration of pregnancy*	*Dose of vasopressin (i/v)*					
		2 U.	*5 U.*	*10 U.*	*15 U.*	*20 U.*	*30 U.*
100	11 weeks		2		3		
39	13 :		0		0		
5	15 :					4	
9	16 :					4½	
3	17½ :		5	7½			
6	18 :			6			
97	18½ :		4				
2	19 :		6½				
37	20 :		10½				
40	Parturition		12				
38	P+12 hrs.	6	8		22½		
98	P+ 2 days		8				
38	P+ 3 days			2			6½
37	P+ 4 days			2			5
1	P+ 5 days			2			

parturition the reactivity was maximal, but this was only slightly greater than in late pregnancy and no dramatic increase in response over the last few days of pregnancy was detected comparable to that observed with oxytocin. The changes for oxytocin and vasopressin are compared graphically in Fig. 8 which indicates that as in the cow the transient but powerful augmentation of oxytocin reactivity is preceded by a less pronounced augmentation of vasopressin reactivity.

DISCUSSION

There are three aspects of these results worthy of comment: the inherent activity of vasopressin in the bovine, the influence of sex hormones on responses to oxytocin and the relationship of each factor to the clinical use of posterior pituitary hormones.

It is known that vasopressin has uterine stimulant activity in some species, but no relevant investigations have been traced in cows or sheep. Our experiments in non-pregnant cows indicate a satisfactory regression between dose and response for both oxytocin and vasopressin solutions, with oxytocin the stronger in all cases. Since the solutions used were commercial preparations prepared by extraction of ox posterior pituitary glands, cross contamination occurs, vasopressin solution containing up to 5 units per cent oxytocin, and oxytocin solution up to 5 units per cent vasopressin. It is therefore necessary to consider whether the responses attributed

Fig. 8

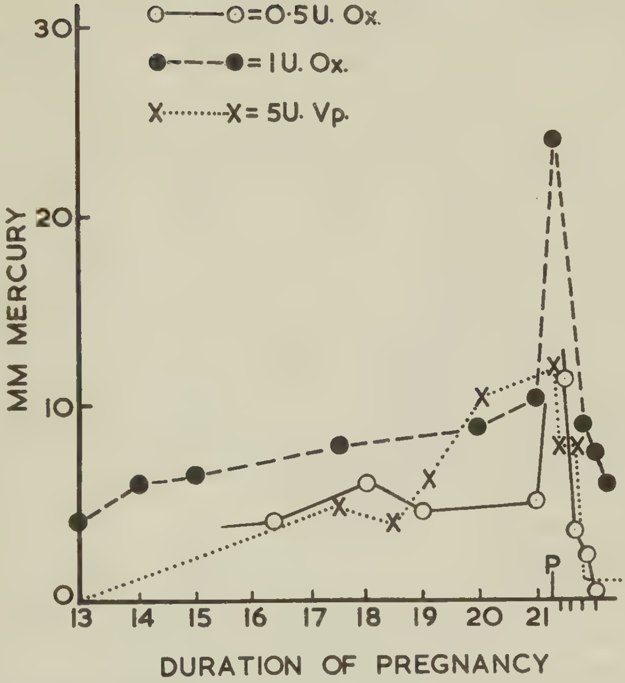

Fig. 8. Uterine responses to oxytocin (OX) and vasopressin (Vp) are compared over the last third of pregnancy in sheep. After the 18th week reactivity to vasopressin increases more rapidly than to oxytocin but at parturition the reverse occurs.

to vasopressin solution are due to the small content of the oxytocin polypeptide. Our own samples of vasopressin solution were kindly reassayed by Dr. G. A. Stewart of the Wellcome Research Laboratories, who reported a figure of 3 units oxytocin per 100 units vasopressin. If the true vasopressor polypeptide has no action on the bovine uterus one would expect our vasopressin solution to have only 3 per cent of the potency of our oxytocin solution, when compared unit for unit. However, our experimental results in non-pregnant cows indicated that vasopressin had a mean potency 17 per cent that of oxytocin and that its activity was greater to a highly significant degree ($P=0.01$) than that which would be accountable on the basis of known contamination with oxytocin. It is concluded therefore that the vasopressor polypeptide has appreciable activity in its own right. This activity of the true vasopressor polypeptide on the bovine myometrium was further exemplified in our cows shortly before parturition and in the puerperium, when vasopressin approached equipotency with oxytocin.

Our observations on the influence of sex hormones are of interest mainly from their relationship to pregnancy and parturition. In the rabbit and in man the onset of pregnancy is accompanied by a marked decrease in sensitivity and reactivity of the uterus to oxytocin. As pregnancy advances lack of responsiveness slowly disappears, but not at the same rate for vasopressin and oxytocin. Near term and at parturition the reactivity to oxytocin is maximal, falling rapidly thereafter. In other laboratory species high reactivity at parturition is known to occur (Reynolds, 1949; Marshall and Moir, 1952). Comparable information on the changes in reactivity of the pregnant uterus of ruminants has not hitherto been available.

Our observations in cows could not be evenly arranged in relation to duration of pregnancy. Nevertheless they indicate that in early pregnancy reactivity to oxytocin is lower than in the same animals when non-pregnant, and that reactivity increased from early pregnancy to parturition. This transition was also seen in sheep in which our observations were spaced more regularly in time. Both in cows and sheep there was a very high reactivity to oxytocin recorded about the time of parturition which developed and faded rapidly.

It is probable that the augmentation in reactivity to both vasopressin and oxytocin at the time of parturition is related to a change from progesterone to oestrogen dominance of the uterus at this time. This is in keeping with the results in the stilboestrol experiments, in which a pronounced increase in reponse to oxytocin and a less marked response to vasopressin were recognised consistently, the augmentation being of the same order as that seen very close to parturition in the same animals.

A change from progesterone to oestrogen dominance of uterine muscle at parturition has been shown in the rabbit (Robson, 1933; Schofield, 1957) and Cross (1958, a, b) has reported the sensitivity of the rabitt uterus to be very similar at parturition and when under oestrogen domination. Increase of oestrogen excretion and change of oestrogen metabolism prior to parturition has been described in man and also in ruminants (Hisaw and Meyers, 1928; Nibler and Turner, 1929; Anderson, 1934; Whitten, 1943; Beck, 1950). Recently, more important evidence has been presented of the critical influence of progesterone block on myometrial activity during pregnancy and of its withdrawal at the end of gestation in the rabbit (Csapo, 1956; Schofield, 1957). The observation of a decline in the concentration of progesterone in the blood of cows shortly before the onset of parturition (Short, 1958) is in keeping with this.

The difference in reactivity to oxytocin and vasopressin noted in our experiments indicates some independent variation in the relative influence of the sex hormones on the sensitivity of the uterus to the two posterior pituitary hormones.

Since vasopressin has an independent uterine stimulating activity the current method of standardising Official (B.P.C. and B.Vet. C.) preparations of Injection of Pituitary (Posterior lobe) Extract may be criticized. At present the potency of such extracts is determined in oxytocic units only, and no indication of vasopressin content is required. It is thus possible for extracts destined for clinical use to contain large but unknown quantities of vasopressin in addition to stated amounts of oxytocin. In Official preparations the implied ratio of pressor to oxytocic activity has always been 1 : 1, but this is not obligatory and there is evidence that in more than 5 per cent of commercial extracts this ratio deviates from unity (Bijlsma, Burn and Gaddum, 1928; Wokes, 1932; Stewart, 1949). Since the only obligatory standardisation is one of oxytocic activity, such deviation from a 1 : 1 ratio will mean an unsuspected variation in vasopressin content in the final product which has been reported as up to 400 per cent (Wokes, 1932). This is important because when used in species giving an appreciable oxytocic response to vasopressin, as in man and ruminants, the original assay figures lose their meaning.

Clinical response may be very irregular consequent upon the side actions of the relatively large dose of vasopressin. These not only involve direct stimulation of uterine muscle, but by causing vasoconstriction at the site of injection and in the myometrium will interfere with the action of the true oxytocin polypeptide. The dangers of fatal vascular shock or coronary constriction due to vasopressin are realised in human obstetrics (Bourne and Burn, 1928; Nixon and Schild, 1953) and purified oxytocin preparations are used almost universally. It is significant that Injection of Pituitary (Posterior Lobe) Extract no longer finds a place in the British Pharmacopoea and has been replaced for obstetrical purposes by Injection of Oxytocin which contains little or no vasopressin. The undifferentiated extract however is described in the 1953 British Veterinary Codex and is undoubtedly the preparation still used by practising veterinary surgeons.

The present usage of posterior pituitary preparations, including oxytocin, may be further criticised on the grounds of dosage. Manufacturers have for many years recommended doses of up to 100 units for cows and mares and this is supported by the 1953 British Veterinary Codex. Such doses fail to take account of the considerable increase in sensitivity at the time of parturition. A dose of 100 units to a parturient cow will represent gross overdosage and if given before delivery carries, at its worst, a risk of death of the foetus or even rupture of the uterus; at its best it is likely to produce incoordination of the uterus and consequent delay in parturition. Jordan (1952) has recorded such incoordination in the form of uterine spasm, lasting up to 19 minutes, in puerperal cows receiving 30 units. It is probable that a realisation of the dangerous and variable nature of uterine response to pituitrin is one factor which has restricted the obstetrical use of oxytocin in ruminants.

It is instructive in this respect to refer to the current use of oxytocin in the species most closely studied, namely, man. High sensitivity at parturition is recognised and the drug is given very cautiously. The official intramuscular dose which is only 2½ units (0.25 ml. of Injection of Oxytocin B.P.) is much less relative to body weight than that recommended for all domesticated species in the 1953 British Veterinary Codex. In man, for elective induction or for the management of early labour, intramuscular injection is being replaced by intravenous infusion of very low dilutions of oxytocin at rates of 1 to 8 milliunits per minute (Theobald, Kelsey and Muirhead, 1956). Responses to such low doses are not dramatically powerful, but are sustained at a magnitude comparable to the contractions of normal labour (Caldeyro-Barcia and Poseiro, 1958) and thus represent a more physiological approach than a single injection. Responses to these infusion doses in parturient women are comparable in magnitude with our observed responses in cows at parturition receiving doses of 1 unit or less intravenously.

In veterinary practice intramuscular injection will remain the most practicable route of administration, at least for some time. Our two experiments on the administration of oxytocin by two routes indicate that doses given intravenously are equipotent to, and of equal duration with, doses 2½ times as great given intramuscularly. It appears that, even when administered intramuscularly, it is difficult to justify the use of doses larger than 10 or at the most 20 units oxytocin in parturient cows, and proportionally less in sheep. Such doses might with discretion be repeated at approximately 30 minute intervals. The doses hitherto recommended appear to be unduly large, and should only be given, if at all, when the foetus has been delivered or at other times when the uterus is empty.

CONCLUSIONS

The reactivity of the bovine myometrium to oxytocin was investigated. In the non-pregnant animal responses are augmented by treatment with stilboestrol. In pregnancy reactivity is low, but develops suddenly to a high magnitude for a brief period at about the time of parturition.

Vasopressin has an inherent uterine stimulating activity in bovines. Variation in uterine response is similar to that with oxytocin but augmentation by oestrogen is less. Augmentation of response at parturition is less than with oxytocin but extends over a longer period. Similar reactions occur with oxytocin and vasopressin in pregnancy and parturition in sheep.

The results are discussed in relation to the secretion of sex hormones and to the inherent activity of vasopressin. The present method of standardising Pituitary (Posterior Lobe) Extract of the British Veterinary Codex is criticized. The use of unfractionated pituitary extracts is considered dangerous and unreliable and the current recommended dosage of preparations containing oxytocin is criticized as being far too high.

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OVARIAN ABNORMALITIES IN THE BITCH

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INTRODUCTION

Pathological studies of the canine female genital tract have been largely concerned with uterine disease; ovarian abnormalities have received little attention. Mulligan (1949) observed 7 ovarian tumours in a survey of 1000 tumours of the dog. Cotchin (1951, 1954) found that only 24 of 2361 canine tumours were of ovarian origin. Bloom (1954) recorded a variety of ovarian tumours in the bitch but gave no indication of incidence. None of these workers gave any indication of the frequency of ovarian tumours in the dog population, nor did they record the incidence of these tumours with respect to age or parity. There is no adequate description of the histological and histochemical features of these canine neoplasms.

This publication is based on the examination of the ovaries of 400 unselected bitches submitted for routine autopsy in the University of Glasgow Veterinary Hospital.

MATERIALS AND METHODS

Each ovary was sectioned through the hilus, fixed in formol-sublimate or neutral formol, dehydrated and cleared in an alcohol-amyl acetate-benzene series and double-embedded in celloidin and paraffin. Routine staining was by haemalum and eosin; other methods used in selected cases were picro-Mallory, Gordon and Sweet's reticulin stain, toluidine blue, alcian blue, Southgate's mucicarmine, sudan black B, periodic acid-Schiff before and after hydrolysis with diastase and the methylene blue extinction test.

RESULTS

Incidence

One hundred and fifteen of the 400 bitches examined showed ovarian lesions, an incidence of 28·75 per cent. The relationship of the various changes to age is shown in Fig. 1. As would be expected these are diseases of adult life and increase with age. Of the 400 animals in the survey, 93 had a total of 127 tumours of various types in one or more sites. As twenty-five bitches had primary ovarian tumours, 27 per cent. of all tumour cases and 20 per cent. of all tumours were ovarian in origin. In only one case was the ovarian tumour considered to be the primary cause of disease. All ovarian tumours were in bitches of 5 years and over. The frequency of each ovarian lesion is shown in Table 1.

Tumours

Granulosa Cell Tumours

Though tumours of granulosa cell origin were found in 13

Fig. 1

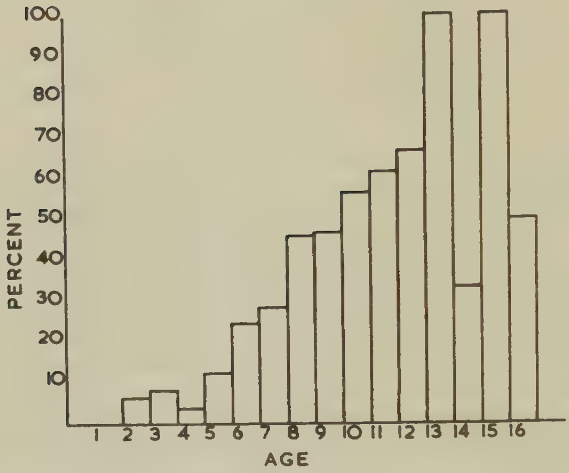

Fig. 1. The age incidence of ovarian abnormalities in the bitch.

animals, only in 9 were the ovaries considered abnormal at autopsy. The bitches ranged from 5 to 15 years and 10 of them were nulliparous. The tumours were commonly round or oval with a smooth or slightly lobulated surface and ranged from 0·4 to 10 cm. in diameter. The smaller tumours were solid on section, but some of the larger ones were soft and friable and contained cystic spaces of various sizes. The cut surface had a granular appearance and was cream or faintly yellow with areas of haemorrhage and necrosis in the larger masses. Metastases were not observed in any of these cases though a microscopic tumour nodule was found in the contralateral ovary of one case. The unaffected ovaries contained corpora lutea in 9 animals and were inactive in the other 4 cases. There was an ascites and hydrothorax of undetermined origin in one animal.

Histology. The predominant cell type in 9 cases was similar to the granulosa cells present in normal medium-sized follicles. The cells were small and polyhedral with a small amount of palely eosinophilic cytoplasm in which there were a moderate number of argentophil granules. The relatively large round or oval nuclei were central in position. They had an abundant chromatin network and a well-defined nuclear membrane. Mitotic figures were rare in most cases.

The histological pattern varied considerably, not only from case to case but also in different fields of individual sections. All were well encapsulated and showed no evidence of local infiltration. The most common arrangement was diffuse fields of granulosa cells in which a variable number of folliculoid structures occurred. These acini were lined by a layer of columnar type cells and contained an eosinophilic fluid. They varied in size from small Call-Exner rosettes to relatively

TABLE 1

AGE INCIDENCE AND LESIONS FOUND

Age	*No. of animals*	*Granulosa cell tumours*	*Serous cystadenoma*	*Pseudo-mucinous cystadenoma*	*Adeno-carcinoma*	*Fibroma*	*Solitary follicular cysts*	*Multiple follicular cysts*	*Lutein cysts*	*Germinal cysts*	*Rete cysts*
1	39	0	0	0	0	0	0	0	0	0	0
2	27	0	0	0	0	0	1	0	1	0	0
3	23	0	0	0	0	0	2	0	0	0	0
4	33	0	0	0	0	0	1	0	0	0	0
5	32	1	0	0	0	0	3	0	0	0	0
6	39	0	1	0	0	0	4	1	1	2	1
7	42	2	0	1	0	0	3	1	1	3	1
8	39	2	1	0	0	1	4	2	2	3	3
9	37	1	2	1	1	0	5	4	0	2	2
10	34	2	2	0	0	0	5	4	1	4	1
11	23	2	0	0	0	0	5	3	1	2	1
12	18	1	1	0	0	0	4	3	1	3	0
13	6	1	1	0	0	0	2	2	1	1	0
14	3	0	0	0	0	0	1	0	0	0	0
15	3	1	0	0	0	0	1	1	0	0	0
16	2	0	0	0	0	0	0	1	0	0	0
	400	13	8	2	1	1	41	22	9	20	9

large cysts (Fig. 2). In one of the tumours there were several large cystic cavities lined by many layers of granulosa cells which were surrounded by a distinct connective tissue capsule. These structures resembled the folliculoma malignum of von Kahlden. The fluid within the cysts was weakly P.A.S. positive, but was not metachromatic and did not stain with alcian blue or with Southgate's mucicarmine. Methylene blue extinction occurred at pH 5.

The connective tissue stroma in the majority of these cases was not conspicuous and reticulin fibres did not penetrate the granulosa cell masses. In 2 cases there were numerous tissue trabeculae extending in from the capsule and splitting up the granulosa cells into round or oval columns. This connective tissue proliferation was very prominent in one case and granulosa cells appeared as small isolated islands or columns. In this cylindromatous pattern, the cells immediately adjacent to the trabeculae were columnar in type and lay on a thin layer of argyrophil fibres. In one specimen of the diffuse cellular type there were areas of transition from typical granulosa cells to plump, fusiform cells resembling those of the theca interna. These thecal cells were arranged in an irregular interlacing manner and were separated by distinct bands of fibrous tissue. Unlike the granulosa cells, they had a well-developed reticulin network and did not have intra-cytoplasmic argentophil granules. Both the granulosa and thecal cells in this tumour contained small numbers of lipid granules.

There was transformation of the cells into typical granulosa lutein cells in 3 of these tumours. In 2 cases this change was focal and occurred mainly in the centre of diffuse cellular masses, but in the third case luteinisation had occurred in the majority of the cells. The transitional stages between granulosa and lutein cells were similar to those observed in the normal follicle during the later stages of maturation. There was a gradual increase in the size and in the lipid content of the cells as the degree of luteinisation increased.

Though hormone assays were not performed on body fluids or on tumour tissue some evidence of ovarian functional activity could be gained from histological examination of the uterus, vagina and skin. The vaginal epithelium was hyperplastic and exhibited a degree of cornification indicative of oestrogenic activity in 3 cases in which the contralateral ovary was atrophic. In these, the endometrium showed slight glandular dilatation and stromal fibrosis similar to that observed following prolonged oestrogen treatment. One of these animals showed atrophy of the epidermis and hair follicles suggestive of prolonged oestrogenic activity. There were 9 cases in which the vaginal and endometrial histology was compatible with metoestrus. In 7 of them the contralateral ovary contained corpora lutea and the other 2 were associated with luteinised tumours. There was no evidence of progestational activity in the endometrium or vagina of the third luteinised tumour.

Serous Cystadenoma

Tumours of this type were found in 8 animals ranging from 6 to 13 years old. Five of them were nulliparous and only one had whelped more than one litter. Six of the tumours were small unilocular structures of 0·8 to 1·5 cm. in diameter and 2 were multilocular masses of 6 to 8 cm. in diameter. Intraluminal papillary growth was prominent in most specimens. The contralateral ovary contained follicular cysts in 3 cases and corpora lutea in 2 cases.

Histology. There was evidence of multicentric origin in all the small tumours with the formation of microscopic papillary cysts in other parts of the ovarian cortex. Papillary cysts were present in the contralateral ovary in 2 cases. The walls of the cysts were composed of dense vascular connective tissue which was hyaline in some of the larger specimens. Arising from this capsule were a number of intracystic papilligerous processes which almost occluded the lumen in some of the smaller cysts. These processes varied greatly in size and configuration. They were commonly slender and villiform with fine connective tissue cores in the smaller cysts. In the larger cysts, the processes were thicker and more irregular in shape. Some were simple papillae with broad cores of oedematous connective tissue, whilst others exhibited a more complex arborescent pattern (Fig. 3). In the latter, numerous acini were included in the substance of the processes, producing an adenomatous appearance. Extracystic growth was not prominent but small solid papillomata were present in 3 cases. Small calcified granules, the so-called psammoma bodies, were present in the stroma of 2 of the tumours.

The lining epithelium was composed of a single layer of cells which varied considerably in type, even in individual cysts. The predominant type of epithelium was composed of cuboidal or low columnar cells with central vesicular nuclei and homogeneous cytoplasm. The epithelium in 2 cases showed certain similarities to that of the oviduct and was composed of tall ciliated columnar cells and intercalary cells with deeply basophilic cytoplasm. A few Schiff-positive granules were present in these tubal-type cells but not elsewhere. Lipid was not present in the epithelium of any of the tumours. The cyst lumina contained pale eosinophilic fluid with an admixture of cell debris and blood in some cases This fluid was Schiff-positive, but was not metachromatic and did not stain with alcian blue or Southgate's mucicarmine. It did not contain lipid.

There was no histological evidence of derangement of the oestrus cycle due to these tumours. The endometrial and vaginal histology was compatible with the functional activity shown by the contralateral ovary.

Pseudomucinous Cystadenoma

Two tumours of this type were found in maiden bitches of 7 and 9 years old. The older animal had shown some evidence of oestral abnormality for almost 2 years. The tumours formed unilateral lobulated masses of 7 to 10 cm. in diameter. On section, each was composed of a number of lobules containing a viscid milky fluid.

The cyst walls were thin and translucent with only occasional papillary growths.

Histology. The tumours were composed of a number of relatively large cysts surrounded by a honeycombed mass of microscopic acini (Fig. 4). The cysts had a well-defined connective tissue capsule containing coarse collagen fibres and dense bundles of elastin. Smooth muscle fibres were not evident in the capsule. The few papillae which projected into the cyst lumina were long and slender. The cyst epithelium rested on an argyrophil basement membrane which was particularly prominent in the small locules.

The cysts were lined by a single layer of very tall columnar cells with clear refractile cytoplasm. The oval nuclei which were deeply basophilic were situated close to the basement membrane. Small nucleoli were evident in many nuclei. The cell membranes were prominent and there were no cilia on the free border. Large goblet cells were numerous in the epithelium of some cysts and absent in others. The cytoplasm of all the epithelial cells contained aggregated masses of Schiff-positive material. This material also stained with mucicarmine and alcian blue and showed *B*-metachromasia with toluidine blue. The cyst lumina also contained fluid of this nature. The cytoplasm of some of the cells also contained a number of minute, finely dispersed lipid droplets.

Adeno-carcinoma

Autopsy of a 9 year old maiden bitch revealed a nodular mass 12×9×7 cm. in the left ovary. On section, the bulk of the tumour was firm and solid, but a cystic cavity of 2 cm. diameter was present in the centre. There was widespread peritoneal dissemination of the tumour with the formation of large solid masses in the omentum and mesenteries. Small discrete metastatic nodules were present in the sublumbar lymph glands, liver and lungs. There was a moderate degree of ascites and hydrothorax.

Histology. The structure of the primary tumour varied in different areas. Long branching papillary processes extended into the lumina of the central cystic portion (Fig. 5) and were covered by a tall columnar epithelium with central deeply staining nuclei similar to those of a serous cystadenoma. The epithelium ranged in height from one to many layers and had a moderate mitotic rate. Some of the central cysts had well-defined connective tissue capsules, but elsewhere the papillary processes sprung from broad fields of solid adenocarcinoma. The major portion of the tumour was less well differentiated and was composed of solid sheets of cells with only occasional acini. The connective tissue stroma was fine and diffusely arranged throughout the tumour. The metastases were of a similar solid pattern with only a few scattered acini. There was a small amount of Schiff-positive fluid in some of the acini and in a few of the surrounding epithelial cells. This material was not metachromatic and did not stain with mucicarmine or alcian blue.

The contralateral ovary contained a number of corpora lutea which were regressing, The endometrial and vaginal histology was consistent with the later stages of metoestrus.

Fibroma

A single rounded mass of 9 cm. diameter was an incidental finding at autopsy in an 8 year old bitch which had borne 3 litters, the last 6 months prior to death. The tumour was solid and hard to cut and had a whitish, homogeneous surface. There were no corpora lutea or follicles in the other ovary and the uterus appeared normal.

Histology. The tumour was composed of interlacing bands of fibrous tissue. The majority of the connective tissue bundles were moderately cellular with a fine collagenous stroma. The cells were fusiform with plump, deeply basophilic nuclei resembling those of the theca interna. Unlike the latter they did not contain lipid or P.A.S.-positive material. In other bundles, the connective tissue was relatively acellular and the collagen fibres were coarse and frequently hyalinised. In such bundles, the cells were small with scanty cytoplasm and elongated dense nuclei. There were no mitotic figures in any part of the tumour and smooth muscle cells were present only in the walls of vessels. Though normal ovarian structures were present in the hilar portion of the capsule, no epithelial elements were present within the tumour. The other ovary contained only primary follicles and remnants of corpora lutea. The endometrial histology was consistent with the terminal stages of metoestrus.

Secondary Tumours

Though 74 of the bitches submitted to autopsy had metastases from tumours arising in a variety of sites, only in 3 cases were secondary deposits found in the ovaries. Gross involvement of both ovaries was present at autopsy in 2 cases with widespread metastases from lymphoid tumours of the small intestine. A microscopic secondary was found in one ovary of an animal with a widely metastasising mammary carcinoma. Lymphosarcoma and mammary carcinoma were the commonest malignant tumours found at autopsy.

Ovarian Cysts

Follicular Cysts

Solitary cysts were found in 41 animals of 2 to 15 years of age and at various stages of the oestrus cycle. Twenty-nine of the bitches were nulliparous and only 7 were multiparous. The majority of the cysts measured 1 to 1·5 cm. in diameter but a few ranged up to 5 cm. On section they were thin-walled, monolocular and contained a clear watery fluid. Corpora lutea at various stages of growth and regression were present in the affected and normal ovaries of 31 cases. There was cystic hyperplasia of the endometrium in 11 cases.

Multiple ovarian cysts were present in a further 22 animals of 5 years and over. Eighteen of these were maiden bitches. The cysts formed masses up to 10 cm. in diameter. The component cysts were commonly small and appeared to be monolocular. There was bilateral involvement in 7 cases. In 5 bitches, there was a concomitant cystic glandular hyperplasia of the endometrium.

Histology. Evidence of follicular origin was readily apparent in the smaller solitary cysts. They were lined by several layers of granulosa cells within a capsule composed of regressing theca and compressed ovarian stroma. The nuclei of the granulosa cells were less regularly shaped than those of the normal follicle and showed complete loss of polarity. Degenerate remnants of the cumulus oöphorus could be seen in many of the cysts. The nuclei became compressed and orientated parallel to the capsule as the size of the cysts increased. The lining of the largest cysts consisted of a single layer of cuboidal or flattened cells. The capsule was dense, fibrous and often hyalinised. Where multiple cysts were present, the smaller ones were lined by a typical granulosa cell layer whilst the larger ones were lined by a low cuboidal epithelium. The epithelium in some of the larger cysts had completely disappeared and they were lined by dense hyalinised connective tissue. All the cysts presented a simple cavity with no evidence of intracystic growth or of communication between individual cysts.

All stages of follicular aad luteal growth and regression were present in the cases with single cysts and in those with unilateral multicystic change but were absent in cases with bilateral involvement. There was no histological evidence of oestrogenic activity in the endometrium or vagina of any of these cases.

Lutein Cysts

Solitary cysts lined by luteal tissue were found in the ovaries of 9 bitches of 2 to 13 years. Six of the animals were nulliparous. The cysts ranged from 1·5 to 3 cm. in diameter and had thicker and more opaque walls than those of the follicular type. The ovaries in all cases also contained normal corpora lutea.

Histology. The normal corpus luteum in the dog shows central cavitation for a short time after ovulation but this rapidly disappears. None of the normal corpora lutea in these cases showed such cavitation. Six of the cysts were completely lined by several layers of granulosa lutein cells arranged on a capsule of compressed ovarian stroma. The lutein cells in the smaller cysts were similar to those of neighbouring normal corpora lutea and were supported by vascular connective tissue trabeculae extending in from the capsule. A thin layer of fibrin was deposited on the inner surface of the layer of lutein cells. The cells lining the larger cysts were less well preserved; the cells on the luminal surface were compressed and many contained pyknotic nuclei. A layer of fine connective tissue separated the lutein cells from the lumen in several of the larger cysts.

Only a portion of the wall was lined by lutein cells in 3 cases, the remainder being lined by granulosa cells like those in follicular cysts (Fig. 6). Luteinisation of the granulosa cells was focal in 2 cases and a gradual transition from one cell type to another could be traced. Similar foci of luteinisation were present in the walls of the cyst in the third case, but the change appeared to be present in the theca interna since the granulosa cell layer continued over the lutein cells without interruption.

All these lutein cysts contained a pale eosinophilic fluid which did not stain with sudan black B or with P.A.S.

The morphology of the uterus and vagina in these cases was consistent with the progesterone phase of the cycle and this, in the presence of normal corpora lutea, gave no indication of the hormonal activity of the cysts.

Germinal Cysts

These cysts were found on microscopic examination of the ovaries of 20 bitches of 6 to 13 years. Eleven of the animals were nulliparous. They were found in otherwise normal ovaries at all stages of the oestrus cycle and also in association with other ovarian abnormalities.

The cysts were microscopic in size, multiple and commonly bilateral. They were present as irregularly round or oval structures in the cortices of ovaries of the senile type in which there were numerous indentations of the germinal epithelium (Fig. 7). They were lined by a non-secretory cuboidal or low columnar epithelium of the germinal type. There was no evidence of cilia or of metaplastic change. The epithelium lay directly on the undifferentiated ovarian stroma with only slight condensation of argyrophil fibres without the formation of a definite basement membrane. The cysts contained a pale eosinophilic fluid which did not stain with sudan black B or P.A.S.

Rete Cysts

Rete tubules were present as a small mass of irregular, anastomosing tubules in the hilar region of over 90 per cent of the ovaries examined. They were lined by a single layer of closely packed columnar cells with large, pale basal nuclei and ragged free borders.

Single microscopic cysts were present near the hilus of the ovary in close association with groups of rete tubules in 9 animals of 4 to 11 years of age. The cysts were unilateral. The smaller cysts were irregular in outline and were lined by an epithelium similar to that of the normal rete tubules. The larger cysts were more rounded and were lined by an epithelium in which the cells showed loss of polarity. The cells were cuboidal in type and the free edge was more even in outline than in the normal tubules. Intra-cystic papillary processes were present in the smaller cysts but the larger ones were essentially monolocular. The fluid in the cysts was eosinophilic and stained pink with P.A.S. The stroma underlying the cysts was composed of dense fibrous tissue and did not contain muscle fibres.

DISCUSSION

Twenty-five primary ovarian tumours were found in a histological study of the genital tract of 400 bitches submitted to routine post-mortem examination. Ovarian tumours represented 20 per cent of all tumours found in the animals under consideration. Both Mulligan (1949) and Cotchin (1951, 1954) observed that 1 per cent of all canine tumours examined by them were of ovarian origin. This disparity in incidence may possibly be explained by the fact that many of the tumours included in the present study were found only on histological examination. The affected animals ranged from 5 to 15 years of age and 18 out of the 25 were nulliparous which suggests an increased incidence of ovarian neoplasms with age and with absence of pregnancy.

The survey suggests that there is a close similarity between the common ovarian tumours in the bitch and in women. Only in the granulosa cell tumours are there obvious structural differences and, as Willis (1953) points out, the canine tumours show greater variations in morphology and some show distinct resemblances to the papillary adenomas. The granulosa cell tumours in the dog show no evidence of malignancy. Histological evidence of oestrogenic activity was found in the endometrium and vagina in only 3 of the 13 tumours of this type examined. Traut and Marchetti (1940) found that 39 out of 54 human granulosa cell tumours showed evidence of hormonal activity. Another major difference from a comparable series of human ovarian neoplasms is the absence of teratomata from the present series though both Storm (1947) and Cotchin (1956) have observed single specimens. It is noteworthy that tumours of the canine testis have not been recorded.

It is interesting to note the low incidence of secondary tumours of the ovary in the bitch. Novak (1952) states that the commonest metastatic tumours of the human ovary arise from primary carcinomas of the pelvic organs and the intestines. Primary carcimoma of these regions is rare in the dog and though there were 18 cases of intestinal lymphosarcoma in the animals examined, only in 2 cases were there ovarian secondaries. Twenty-nine of the animals had carcinomata of the mammary glands with pulmonary metastases, yet ovarian involvement was present in only one case. Willis (1953) found a similarly low incidence of ovarian metastases from widely disseminated mammary carcinomas in women.

Non-neoplastic cysts of the ovaries were found in 90 animals. Follicular cysts were the commonest and were found in 16 per cent of the ovaries examined. There was no histological evidence of oestrogen activity in any of the cases of follicular cysts. The incidence of all types of ovarian cysts was higher in nulliparous animals and in those over 5 years of age.

CONCLUSIONS

A histological examination of the ovaries of 400 bitches, submitted to routine post-mortem, revealed primary tumours in 25 cases, secondary tumours in 3 cases and non-neoplastic cysts in a further 90 animals. A detailed histopathological description of the individual lesions is given in addition to an examination of their secretory activity with regard to mucin and lipid. Analysis of age incidence suggests that both tumours and cysts of the bitch are more common in animals over 5 years of age and in nulliparous animals.

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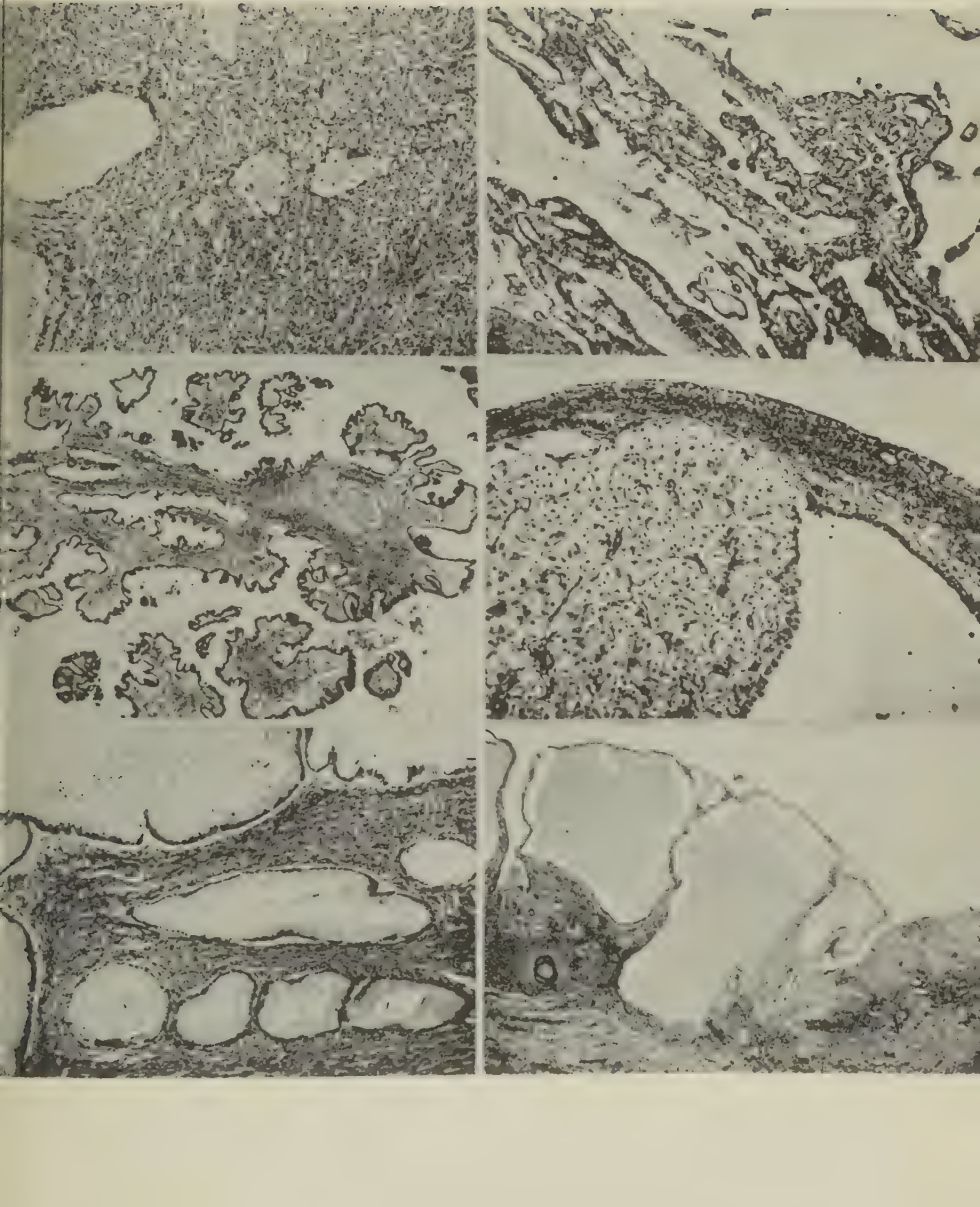

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LEGENDS

Fig. 2. Granulosa cell tumour showing follicular structures of various sizes. H and E × 65.

Fig. 3. Serous cystadenoma showing thick branching intracystic papillary processes· H and E × 20.

Fig. 4. Pseudomucinous cystadenoma composed of numerous cysts of varying size. The cysts are lined by a characteristic tall columnar epithelium with clear refractile cytoplasm. H and E × 65.

Fig. 5. Adeno-carcinoma showing long branching papillary processes extending into the lumen of the central cystic portion. H and E × 65.

Fig. 6. Simple ovarian cyst lined partly by granulosa cells and partly by luteal cells. H and E × 65.

Fig. 7. Group of germinal cysts in the cortex of the ovary. They are lined by a low cuboidal epithelium. H and E × 20.

THE
HISTOPATHOLOGY OF THE SPINAL CORD IN SCRAPIE DISEASE OF SHEEP

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INTRODUCTION

A detailed histological examination of the spinal cord in naturally occurring scrapie disease of sheep is not recorded in the literature.

Several investigators have noted abnormalities in the cord while engaged in more general observations on the whole, or more detailed work on other parts, of the central nervous system (Besnoit and Morel, 1898; Stockman, 1926; Bertrand, Carré and Lucam, 1937; Brownlee, 1940; Plummer, 1946; Lucam, Béchade and Saurat, 1950; Wagner, Goldstein, Doran and Hay, 1954; Palmer, 1957[2]; Zlotnik, 1958[1]).

All the above workers are agreed that some involvement of the neurones may occur although opinions have varied as to whether this consists merely of vacuolisation or a more profound alteration. No attempt has been made to determine whether these lesions are constantly present in the disease. Glial activation, perivascular infiltrations and meningeal lesions have been thought significant by some investigators, while others have failed to observe these appearances. Similarly, considerable importance has sometimes been attached to the presence of eosinophilic intracytoplasmic inclusions, while, at other times, their specificity for scrapie disease has been denied. The occurrence of abnormalities in the spinal cord, and certainly the importance of vacuolisation, have not met with universal acceptance, for both in the past and in recent times hypotheses have been put forward suggesting that lesions of the skeletal musculature are of greater significance (McGowan, 1914; Bosanquet, Daniel and Parry, 1956; Delez, Gustafson and Luttrell, 1957).

This brief review of the literature suggests that it is necessary to investigate the histopathology of the spinal cord in scrapie with particular reference to the significance, constancy and distribution of the lesions.

MATERIALS AND METHODS

The material consisted of the spinal cords of 10 sheep affected with naturally occurring clinically advanced scrapie, 10 sheep with naturally occurring non-advanced scrapie, 16 sheep affected with experimentally induced scrapie and 18 apparently healthy sheep. The criteria upon which the designations "advanced" and "non-advanced", were based were mainly symptomatic, as the length of illness and history were in some cases unknown and in others unreliable. The non-advanced cases were kept under observation only long enough to establish a clinical diagnosis of scrapie; at the time of destruction they were affected with little more than slight pruritus, and in some cases mild ataxia. The advanced cases were all affected at the time of destruction with ataxia and inco-ordination and had at some time shown varying degrees of pruritus. The experimental group of sheep all showed clinical signs of scrapie. This group contained 11 hoggs inoculated intracerebrally at 6 months to 8 months of age and killed when 12 to 18 months old, 2 lambs inoculated intracerebrally at birth and killed when 6 months old, and 3 cast ewes killed about 9 months after subcutaneous inoculation. The inoculum was a suspension of pooled brains obtained from the serial passage of scrapie brains which originated from the successful transmission experiments of Wilson, Anderson and Smith (1950). The age range of the scrapie sheep was covered by the control group of sheep, the majority of which were between 2 and 4 years old, although 5 were more than 7 years and 3 were 8 months old.

All animals were killed by venesection and the cords fixed as soon after death as possible in 10 per cent. formol saline for 1 month. Transverse pieces of cord from the fourth, sixth, seventh and eighth cervical, first and sixth thoracic, third, fifth and sixth lumbar, and first sacral segments, were post-fixed for 48 hours in corrosive formol, dehydrated in alcohols, cleared in benzol and embedded in paraffin. Routinely, sections, 7μ thick, were stained with haematoxylin and eosin, but a variety of staining methods were used for specific purposes such as cresyl fast violet and eosin methylene blue for Nissl substance, Mallory's phosphotungstic acid for glia fibrils, Holmes' methods for axis cylinders and intracellular neurofibrils, Lendrum's phloxine-tartrazine, Gomori's aldehyde fuchsin, Feulgen's method, and the periodic acid-Schiff reagent, picro-Mallory, Heidenhain's iron haematoxylin, Giemsa for intracytoplasmic inclusions, and safranin O-eriocyanine A for degenerating neurones.

Vacuolated neurones were counted with the 4 mm. objective in 20 consecutive 7μ serial sections from each of the 10 segments of the spinal cord listed above. Two hundred sections were thus examined from each of the 20 cases of natural scrapie, the 16 cases of experimental scrapie and 10 control animals. At the same time the location of the vacuolated neurones was determined by arbitrarily dividing the grey matter into 3 zones. Zone A comprised all the neurones ventral to the subs. gel. rolandi and dorsal to the large neurones which lie in relatively well defined nuclei in the ventro-lateral and ventro-medial aspect of the ventral horn. The majority of the latter are motor neurones and they were designated Zone B. The final Zone, C, consisted of the nucleus intermedio-lateralis where it formed a well defined horn (T_6 and L_3).

The number of neurones containing phloxinophilic bodies larger than 2·5μ diameter was counted in 4 serial sections from each of 5 segments of the cord (C_4, C_8, T_6, L_3 and L_6) in 15 natural cases of scrapie, 10 experimental ones and 15 control animals.

Astrocytes were demonstrated by the Cajal method in sections from the cervical and lumbo-sacral intumescentia and the mid-thoracic region. Transverse and longitudinal sections from the mid-cervical and mid-thoracic regions and the cervical and lumbo-sacral intumescentia were stained by the Sudan IV technique and Spielmeyer's method to demonstrate degenerating myelin, and the Bielschowsky-Glees method for degenerating axons. The longitudinal sections were taken at three depths so as to include the ventral, lateral and dorsal funiculi. Seventeen natural scrapie animals 12 experimental scrapie and 17 control animals were examined by these methods.

RESULTS

Grey Matter

Degeneration of neurones. Various forms of degeneration of neurones ranging from slight central chromatolysis to complete necrosis were seen in all cases of natural scrapie. Chromatolysis was seen at all stages, from early central chromatolysis to complete dissolution of the Nissl granules with eccentricity of the nucleus and moderate swelling of the cell body. Acute swelling was not frequent, but severe cell change when the nucleus was pyknotic, the cytoplasm foamy and often eosinophilic, and the neurofibrils disintegrated and clumped, was relatively common. Sclerotic neurones, often with fine peripheral vacuoles, and necrotic neurones were both observed.

In the severe cases, large numbers of all the above forms of degeneration could be seen in any single section, usually together with large numbers of vacuolated neurones, but in some of the less severe cases it was often necessary to examine as many as 10 consecutive sections in order to find one degenerating cell. The neurones of the subs. grisea centralis, the posterior and anterior cornu commissuralis nuclei, and the cells of the intermediate zones were most affected. Degenerative changes were seen in the nucleus intermedio-lateralis in 95 per cent of the scrapie cords, and were sometimes marked in this nucleus when relatively slight elsewhere. The large motor neurones of the ventral horns rarely showed degenerative changes.

Similar forms of degeneration were seen in the same areas of the grey matter in the experimental animals, but were very rare, although occasional chromatolytic cells were seen in every case. At least one or two chromatolytic neurones were observed in the nucleus intermedio-lateralis in 75 per cent of the experimental animals.

Diseased neurones were rarely seen in the spinal cords of the control group of sheep, although in one particular case 5 cells in the 200 sections showed central chromatolysis and 2 showed severe cell change.

Vacuolisation of neurones. Vacuolated neurones were seen in all the cases of natural scrapie and in 15 of 16 experimental animals. The vacuoles were either single or multiple and their size varied from small structures which did not noticeably distort the outline of the cell body to extremely large unilocular or multilocular structures. The majority of vacuoles were situated in the cell body, although in some neurones the cell processes only were vacuolated. In the latter cases the vacuoles were either small or were large enough to cause extreme ballooning of the process. The cytoplasm of vacuolated neurones was either unaltered or showed various

TABLE 1

ADVANCED SCRAPIE GROUP

Vacuolation of Neurones in the Spinal Cord

Case no.	*Average number of vacuoles per 20 sections in regions of the cord*									*Total no. of vacuoles in 200 sections from the four regions of the cord*
	Mid-cervical region C_4		*Cervical intumescentia* $C_6\ C_7\ C_8\ T_1$		*Thoraco-lumbar region* $T_6\ L_3$			*Lumbo-sacral intumescentia* $L_5\ L_6\ S_1$		
	Zone A	*Zone B*	*Zone A*	*Zone B*	*Zone A*	*Zone B*	*Zone C*	*Zone A*	*Zone B*	
NW39 ..	253	65	1054.0	106·25	256·0	35·5	15·5	354·3	100·0	6936
NW10 ..	102	—	193·0	8·5	63·0	4·5	1·0	296·6	10·6	1967
NW13 ..	119	9	270·75	17·25	50·0	2·5	16·5	147·3	13·0	1899
NW33 ..	44	—	112·75	2·0	54·0	—	2·5	82·0	2·0	868
NW23 ..	81	6	78·0	1·75	101·5	1·5	14·0	61·0	3·0	832
NW30 ..	26	2	66·0	—	21·0	—	1·5	81·0	1·0	583
NW9 ..	20	—	46·0	1·5	28·0	1·5	3·0	62·6	—	463
NW18 ..	5	—	46·5	0·25	10·5	—	4·0	69·0	1·6	433
NW36 ..	11	—	31·0	—	10·0	—	3·0	10·0	0·3	192
NW31 ..	8	—	15·25	1·25	5·5	0·5	3·5	23·0	0·3	163

TABLE 2

NON-ADVANCED SCRAPIE GROUP

Vacuolation of Neurones in the Spinal Cord

Case No.	*Average number of vacuoles per 20 sections in regions of the cord*									*Total no. of vacuoles in 200 sections from the four regions of the cord*
	Mid-cervical region C_4		*Cervical intumescentia* $C_6\ C_7\ C_8\ T_1$		*Thoraco-lumbar region* $T_6\ L_3$			*Lumbo-sacral intumescentia* $L_5\ L_6\ S_1$		
	Zone A	*Zone B*	*Zone A*	*Zone B*	*Zone A*	*Zone B*	*Zone C*	*Zone A*	*Zone B*	
NW76 ..	72·0	—	367·5	13·0	38·0	1·5	1·0	282·3	27·0	2603
NW38 ..	17·0	—	21·5	—	32·5	2·5	6·0	55·0	1·3	354
NW42 ..	2·0	—	29·75	1·25	9·5	—	20·0	17·3	—	237
NW26 ..	9·0	—	17·0	—	11·5	—	1·0	32·0	—	198
NW46 ..	6·0	—	7·75	0·25	14·5	2·5	14·0	3·6	0·3	112
NW24 ..	12·0	—	8·5	—	17·0	—	2·5	5·6	0·3	103
NW22 ..	4·0	—	3·25	—	14·0	—	5·0	11·0	—	88
NW29 ..	1·0	—	2·0	—	3·5	—	13·0	2·6	—	50
NW27 ..	2·0	—	6·0	0·5	5·5	0·5	2·0	—	—	44
NW15 ..	4·0	—	2·25	—	—	—	—	2·3	—	20

forms of degeneration. In some cells a necrotic eosinophilic rim around the vacuoles was all that remained of the cell body. As a rule the structure of the nucleus was not altered unless actual degeneration of cytoplasm had taken place. Neurofibrils were often normal in the cytoplasm of vacuolated cells although slightly denser round the edges of the vacuole. They were never present within the vacuoles. In some cells disintegrated neurofibrils were seen in the form of argentophilic staining dust in small isolated areas or around the edge of the smaller vacuoles.

In all cases of natural and most cases of experimental scrapie, some vacuoles contained masses of palely eosinophilic material, sometimes spherical in shape and often situated at the periphery of the cavity. This material was not phloxinophilic when differentiated according to Lendrum's original method, and was P.A.S. negative, although small numbers of P.A.S. positive granules were occasionally seen in some vacuoles.

Two thousand sections from 10 control sheep were carefully examined and a total of only 4 vacuoles, an average of 0·002 vacuoles per section, each in a different animal, were seen. In 3 neurones these vacuoles were small and single and the cytoplasm was otherwise healthy, while the fourth neurone was sclerotic and contained a rather large peripheral vacuole. Details of vacuolation of neurones in the 3 groups of scrapie animals are given in Tables 1 to 3. It can be seen that while in the advanced natural scrapie groups vacuoles were fairly numerous with an average of 7·2 per section (range 0·8 to 34·6), in the non-advanced scrapie sheep vacuoles were less often found with an average of only 1·9 per section (range 0·1 to 13), while in the experimental animals there were only a few vacuoles, averaging 0·12 vacuoles per section (range 0 to 0·35).

The distribution of vacuolated neurones in the various levels and zones of the spinal cord was as follows. (a) The majority of the vacuoles, irrespective of the scrapie groups, occurred in the intumescentia, both cervical and lumbo-sacral. (b) From 88 to 92 per cent of the vacuoles were found in Zone A. All distinguishable nuclei were affected to some degree, but the neurones of the cornua commissuralis anteriores and posteriores, the substantia grisea centralis and the intermediate zones were more severely vacuolated. (c) Zone B, which contains the motor neurones, was as a whole, very little affected, containing only 7·8 and 4·2 per cent of all vacuoles in the advanced and non-advanced natural scrapie groups respectively. In the experimental sheep 6 per cent of vacuoles were present in Zone B, mainly due to the relatively large proportion of vacuoles occurring in the motor area in one sheep. (d) In Zone C, although only a relatively small group of neurones restricted to the thoraco-lumbar region were affected, they contained a large per-

TABLE 3

EXPERIMENTAL SCRAPIE GROUP

Vacuolation of Neurones in the Spinal Cord

Case no.	*Average number of vacuoles per 20 sections in regions of the cord*									*Total no. of vacuoles in 200 sections from the four regions of the cord*
	Mid-cervical region C_4		*Cervical intrumescentia* C_6 C_7 C_8 T_1		*Thoraco-lumbar region* T_6 L_3			*Lumbo-sacral intumescentia* L_5 L_6 S_1		
	Zone A	*Zone B*	*Zone A*	*Zone B*	*Zone A*	*Zone B*	*Zone C*	*Zone A*	*Zone B*	
W20 ..	3	—	8·5	—	2·5	—	1·5	8	—	69
W21 ..	2	—	2·5	—	2·5	—	1·0	4·3	—	32
W24 ..	2	—	3·75	0·5	0·5	—	0·5	3·7	—	31
W17 ..	—	—	2·5	—	—	—	—	7·3	1	35
W18 ..	3	—	0·5	—	—	—	0·5	5·3	—	22
W13 ..	—	—	2·75	0·5	1·5	—	—	4·0	—	28
W23 ..	1	—	2·5	—	1·5	—	0·5	1·0	—	18
W25 ..	—	—	2·0	—	—	—	1·5	2·66	0·33	20
W22 ..	—	—	0·5	0·25	3·5	—	—	3·0	4·3	32
W31 ..	1	—	0·25	—	—	—	—	0·33	—	3
W19 ..	—	—	—	—	—	—	—	—	—	0
W28 ..	5	—	5·75	—	1	—	3·5	5·6	—	54
W29 ..	—	—	0·75	—	0·5	—	2·0	1	—	11
W30 ..	—	—	0·25	—	0·5	—	—	1	—	5
W32 ..	1	—	0·75	—	—	—	—	0·66	—	6
W34 ..	—	—	2·25	—	—	—	—	—	—	9

centage of vacuoles. In advanced scrapie the vacuoles comprised 9·8 per cent of the total number found in the region, in non-advanced scrapie 29 per cent, and in experimental scrapie 44 per cent. As noted earlier the neurones of this zone, which is the intermedio-lateral nucleus, are frequently affected by other degenerative changes.

Cytoplasmic granules. Lipochrome granules and phloxinophilic bodies were seen in the cytoplasm of neurones in both scrapie and control sheep. The lipochrome granules were faintly yellow in unstained sections, were well stained by Gomori's aldehyde fuchsin, were P.A.S. positive and stained brick red with Sudan IV in both frozen and paraffin sections. They stained faintly with Schiff without hydrolysis, but not with eosin or by Lendrum's phloxine-tartrazine method. These granules were most prominent in the 5 very old control sheep, but otherwise there was no difference in their occurrence in control and scrapie animals.

The phloxinophilic bodies were red when stained with Giemsa, picro-Mallory and eosin-methylene blue, and were stained by Heidenhain's iron haematoxylin. They were negative by the following staining methods: Feulgen, P.A.S., Gomori's aldehyde fuchsin and Sudan IV in both frozen and paraffin sections. These bodies were usually spherical in shape and their size varied from less than 0·5μ up to 5μ in diameter. Small bodies of less than 0·5μ diameter were found in all groups of sheep, but it appeared as if larger phloxinophilic bodies were unusually numerous in some, but not all, of the scrapie group. Thus in 2 out of 15 control sheep examined only 2 neurones contained inclusions larger than 2·5μ in diameter, while in 10 out of 15 natural scrapie sheep a total of 132 cells contained these large inclusions. The bodies were rare in vacuolated cells and their frequency bore no obvious relationship to severity of vacuolation or clinical symptoms. Furthermore, in the experimental disease large inclusions were seen in only 7 cells from the cords of 2 out of 10 sheep examined.

Glial elements. Neither an over-all increase in glial elements nor massive generalised neuronophagia was present in any of the scrapie cases, and there was no obvious increase in perineural satellites. The Cajal gold chloride method revealed neither clasmatodendrosis nor gliosis. However, severely degenerated neurones sometimes showed evidence of neuronophagia by one or a small number of microglia. Neuronophagia did not appear to be associated with vacuolated cells unless they were severely diseased, so that it seemed as if vacuolation *per se* did not stimulate phagocytosis.

Large and small phloxinophilic inclusions were infrequently seen in the nuclei of astrocytes of both the control and scrapie sheep. The smaller inclusions were often surrounded by a halo, whereas the larger bodies distended the nucleus to 2 or 3 times its normal diameter. Occasionally P.A.S. positive material was seen in the cytoplasm of glial cells in both control and scrapie sheep.

Vascular system and the meninges. Perivascular infiltrations by cells resembling lymphocytes were present in 3 control animals, in 5 experimental cases of scrapie and in 6 advanced and 5 non-advanced natural cases of scrapie. In 14 of the scrapie animals infiltrations were infrequent and did not differ in degree or extent from those noted in the control sheep. They consisted at the most of 3 layers of infiltrating cells or a small focal accumulation along the vessel wall, and they were present in the grey or in the white matter close to the grey matter. In one advanced and in one non-advanced scrapie animal more extensive changes were visible in the form of moderately frequent perivascular cuffings.

Occasional fat-laden glia cells were seen close to a blood vessel in the grey matter of 4 cases of scrapie, and paradventitial deposits of material resembling lipochrome were seen in both scrapie and control animals. Small subpial infiltrations of leucocytes, involving only a limited region of the meningeal circumferences, were seen in one advanced and 3 non-advanced cases of natural scrapie. No meningeal lesions were seen in the experimental disease.

White Matter

In 2 natural cases of scrapie definite degeneration of myelin and axis cylinders in the medial region of the ventral funiculus was demonstrated by the methods of Bielschowsky, Sudan IV and Spielmeyer. In one of these cases the dorsal funiculus was also involved, but in the upper cervical region only. In transverse sections the changes in the former two funiculi were seen to extend from the cervical to the lower lumbar region. In longitudinal sections the degenerated myelin sheath was seen in the form of myelin ovoids, while myelophages laden with neutral fat lay both in and outside the digestion chambers. The axis cylinders were considerably swollen and covered with argentophilic granules, or were beaded and vacuolated, or broken into fragments. There was an obvious loss of axons due to their complete destruction, but healthy fibres could be seen amongst the degenerated.

In 6 other natural scrapie animals and in 1 control sheep discrete myelin and axonal lesions were present. The type of lesion and their distribution were similar to the preceding 2 cases, but very seldom were more than a few fibres in each longitudinal section affected. Neutral fats could be demonstrated in all these cases.

In a further 6 natural scrapie cases and in 6 control animals slight myelin changes were observed, but in no case could neutral fat be demonstrated. In all these cases occasional chains of myelin ovoids were seen in longitudinal sections, and in Sudan IV preparations these ovoids were orange-red in contrast to the brick-red coloration of normal sheep myelin. In some of the ovoids, haematoxylin staining showed myeloclasts without fat droplets. Although these lesions were as a whole rather rare, the general impression was that the chains of myelin ovoids were more frequent, particularly in the medial aspect of the ventral funiculus, in the scrapie cases than in the control animals.

Significant changes could not be detected in the white matter in the 12 cases of experimental scrapie, in the remaining 3 cases of natural scrapie or in the 10 control sheep.

DISCUSSION

The results of the present study show that the most constant lesions in the spinal cord from both natural and experimental scrapie cases are degeneration and vacuolation of neurones. In this respect the results are similar to those in the medulla as recorded by many workers (Bertrand *et al.*, 1937; Holman and Pattison, 1943; Wagner *et al.*, 1954; Palmer, 1957[2]; Zlotnik, 1958). The present study does not, however, support the work of Brownlee (1940), who found no lesions, other than large vacuoles, in apparently healthy neurones of the spinal cord.

Careful examination of cords from healthy sheep revealed that neuronal changes, including vacuolation, are so rare that they may almost be ignored. The number of vacuoles in the diseased cords was lowest in experimental scrapie and highest in advanced natural scrapie, which is very similar to that observed in the medulla (Zlotnik, 1958[1], 1958[2]). Bosanquet *et al.* (1956) and Parry (1957), found only occasional vacuoles in the cord which they considered of little significance because of their presence in normal sheep. This opinion is not supported by the present study, for vacuolated neurones were found extremely rarely in the healthy animals while increasing numbers of vacuoles were found in the 3 groups of scrapie sheep.

According to Bertrand *et al.* (1937) and Brownlee (1940) the large neurones of the anterior horns were principally affected in scrapie, and Lucam *et al.* (1950) specifically recorded that the motor neurones were affected. Wilson *et al.* (1950) could not find any vacuoles in the large neurones of the spinal cord in experimental scrapie, but they do not state whether vacuoles occurred in small and medium sized neurones. The present results, however, show that only a very small proportion of vacuoles were found in the motor areas where the largest cells of the cord occur (Zone B; 4·2 to 7·8 per cent). This finding confirms the suggestion of Cassirer (1898) that on a symptomatic basis the motor protoneuron may be excluded, and it also explains the fact that neither Bosanquet *et al.* (1957) nor Hulland (1958) found any evidence of motorneuronal patterns in the skeletal muscles.

The majority of lesions, both vacuoles and various forms of degeneration, were present in Zone A. This zone contains in the main small and medium sized cells interposed between the incoming dorsal root fibres, or fibres descending from higher centres, and the motor neurones. Lesions in these intercalated neurones may be one of the factors causing inco-ordination in scrapie by interference with the passage of ipselateral and contralateral reflexes, involvement of the nuclei cornu commissuralis and the neurones of the substantia grisea centralis particularly suggesting that contralateral reflexes are often affected.

Bertrand *et al.* (1937) suggested that central lesions of the autonomic nervous system may account for the pruritus seen in scrapie: whether or not this is so, the present survey shows that the intermedio-lateral nucleus (Zone C), which contains the preganglionic neurones of the sympathetic nervous system was frequently and sometimes severely affected. Some of the less characteristic symptoms of scrapie suggest autonomic involvement, and it is possible that severe degeneration of the preganglionic neurones may severely undermine the animals' ability to maintain homeostasis.

Eosinophilic intracytoplasmic bodies were described in the neurones from scrapie sheep by Stockman (1926) who considered them significant. Similar or identical bodies have been reported by Brownlee and Wilson (1932) in apparently healthy sheep and in those affected by louping-ill. Palmer (1957[1]) reported eosinophilic structures inside vacuoles and in the cytoplasm of neurones in scrapie sheep, while Zlotnik (1957[1], 1958[1]) found eosinophilic bodies in both healthy and scrapie animals. In the present study, phloxinophilic bodies were found in the neurones of the cord from both healthy and scrapie sheep, although in a proportion of cases of the natural disease increased numbers of large inclusions were present. The presence of these bodies in healthy sheep makes it impossible to consider them specific for scrapie, and the absence of the large bodies from the majority of the experimental cases and many of the natural cases suggests that they have little significance in the disease. However, their increase in some cases of scrapie may be due to altered neuronal secretion or metabolism in the disease.

Although slight neuronophagia of some degenerated neurones was observed in the scrapie cases, glial changes in the spinal cord were not a prominent feature of the disease and astrocytic proliferation was completely absent. In the latter respect the results do not agree with those of Bertrand *et al.* (1937) who reported an astrocytic gliosis in the anterior horns of the cord. Astrocytic proliferation has been reported in higher centres, especially the medulla, by Bertrand *et al.* (1937), Wagner, *et al.* (1954) and Zlotnik (1958[1]). Palmer (1957[2]) could find no astrocytic changes in the brain.

Bertrand *et al.* (1937) recorded that perivascular infiltrations were rare in scrapie cases when these were destroyed in the terminal stages of the disease. Wilson *et al.* (1950) described lesions of subacute meningoencephalitis with perivascular infiltrations in experimental scrapie. Zlotnik (1958[1]) recorded that of 3 groups of advanced scrapie, non-advanced scrapie and healthy sheep, respectively, examined by him, the advanced scrapie were the least affected and the healthy sheep the most severely affected. Zlotnik (1958[2]) found no difference in vascular changes in experimental scrapie and healthy sheep and did not consider them significant. In the present study the incidence and severity of vascular lesions were more or less similar in both advanced and non-advanced natural scrapie groups. In healthy animals and experimentally infected sheep vascular changes were less frequently seen than in the natural disease, but the difference in incidence and severity in all 4 groups of sheep was so slight that it is doubtful if much significance can be attached to these lesions in the spinal cord.

Secondary degeneration of fibres in the white matter was marked in 2 cases of scrapie, but on the whole the positive cases of scrapie showed no more than occasional degenerating fibres discretely scattered in the ventro-medial part of the ventral funiculus and to a lesser extent in the lateral funiculus. This absence of tract degeneration is in agreement with the findings of Cassirer (1898), Stockman (1926) and Brownlee (1940). These authors suggested that occasional degenerating fibres may occur although, because of the capriciousness of the Marchi method in the sheep, these findings were not considered significant. Although age and chronic wasting conditions tend to cause small numbers of fibres to degenerate (Duncan, 1931), the peculiar localisation of the fibres in the scrapie cases suggests they are not due to these incidental factors. King (1911) observed degenerating tracts in similar regions subsequent to experimental medulla lesions in the sheep, and as the neurones of the brain stem are definitely involved in scrapie (Zlotnik, 1958[1]), it is possible that the degeneration seen in the spinal white matter may be secondary to these lesions.

CONCLUSIONS

Vacuolated neurones were either absent or extremely rare in the spinal cords of healthy sheep.

The most significant lesion in the spinal cord in scrapie was vacuolation and degeneration of neurones.

All animals affected with the natural disease show neuronal changes in their spinal cords. In the experimental disease lesions were seen in only 94 per cent of animals.

Vacuolisation of neurones in the natural cases bore a more or less significant relationship to the severity of the clinical symptoms.

The most commonly affected cells were the intercalated neurones and the central neurones of the sympathetic system, while the motor neurones were relatively infrequently affected.

Intracytoplasmic phloxinophilic bodies were observed in both healthy and scrapie sheep. However, in some of the natural scrapie cases, large phloxinophilic bodies were unusually numerous.

Increased satellitosis and massive neuronophagia were rare, but occasional degenerated cells showed evidence of neuronophagia.

Secondary degeneration affecting both axis cylinders and myelin was found in the ventral and lateral funiculi. Usually few fibres were affected, and in only 2 cases was the degree of degeneration marked.

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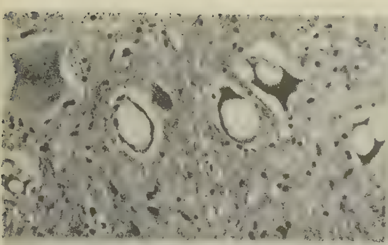
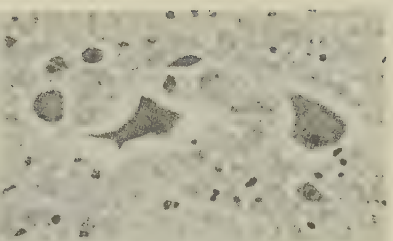


Fig 1



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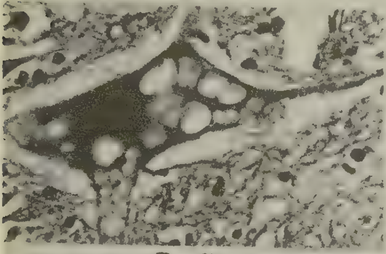

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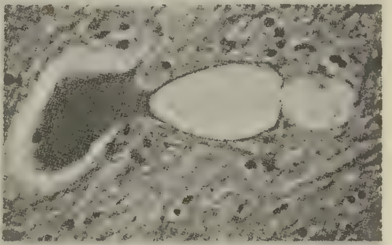

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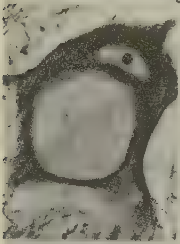
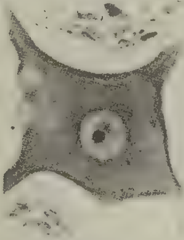


Fig 5a



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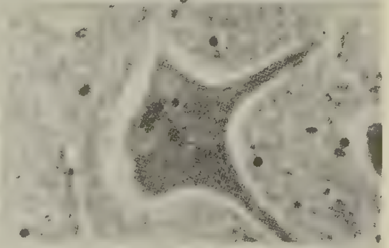
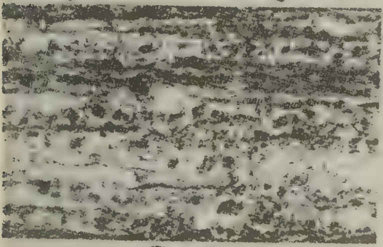


Fig 6



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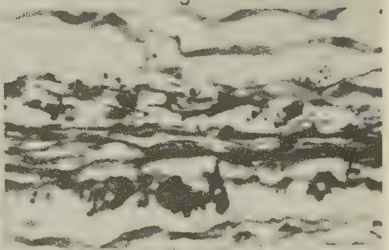

Fig 8

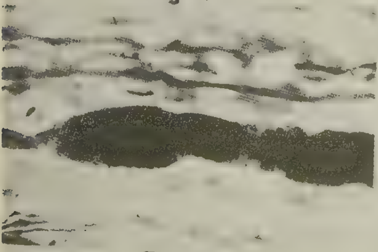

Fig 9

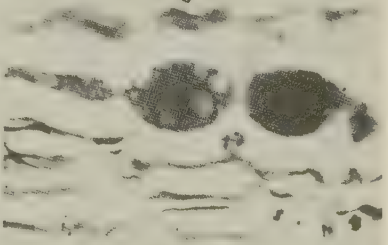

Fig 10

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Vacuolated neurones and neurone showing early acute swelling in the intermediate zone. H and E × 130.
Fig. 2. Degenerating and chromatolytic neurones in the intermediate zone. H and E × 130.
Fig. 3. Multilocular vacuolisation of a neurone and phagocytosing glia cells. H and E × 320.
Fig. 4. Vacuolisation of a process of a neurone. H and E × 320.
Fig. 5. (a) Vacuolated neurone with apparently normal neurofibrils elsewhere in the cell cytoplasm. (b) Early vacuole formation. Holme's method × 330.
Fig. 6. Phloxinophilic inclusions in the cytoplasm of a neurone. Phloxine/Tartrazine. × 320.
Fig. 7. Digestion chambers and myelin ovoids in the ventral funiculus. Spielmeyer's method × 80.
Fig. 8. Fragmented axons in the ventral funiculus. Bielschowsky method × 320.
Fig. 9. Swollen axon in the ventral funiculus showing granular argentophilic deposit Bielschowsky method × 320.
Fig. 10 Beaded swelling and vacuolation of an axon in the ventral funiculus. Bielschowsky method × 320.

STUDIES ON SOME CHEMICAL ASPECTS OF THE PATHOLOGICAL ACTIVITIES OF STRAINS OF *ESCHERICHIA COLI* OF BOVINE ORIGIN

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INTRODUCTION

In recent years certain strains of *E. coli* have been found to be associated with pathological conditions both in man and animals, particularly with diseases affecting the gastro-intestinal tract of the young mammal (Thomson, 1955; Lovell, 1937; Rees, 1958). The chemical basis of diseases associated with increased numbers of *E. coli* has yet to be elucidated. Although toxins have been isolated from *E. coli* strains by many workers (Boivin and Mesrobeaunu, 1936a, 1936b; Westphal, Luderitz, Eichenberger, Keiderling, 1952) the relationship between toxicity and pathogenicity of individual strains has not been clearly established.

The chief toxic component of smooth strains of *E. coli* is a polysaccharide—phospholipide complex, possibly with a protein component. It is attached to the bacterial bodies, of which it appears to be an integral part, and seems to conform to the classical definition of an endotoxin (Topley and Wilson, 1955). Thomas (1954), reviewing the physiological effects of lipopolysaccharide endotoxins, noted that a wide range of Gram-negative endotoxins have similar chemical, physical and biological properties. These include high molecular weight, an apparent identity with the 'O' somatic antigen, and lethality for small laboratory animals. Less than 1 μg of *E. coli* toxin, administered intravenously, is pyrogenic for rabbits and man (Westphal *et al.*, 1952). Very small amounts are able to induce haemorrhage in tumours (Ikawa, Keopfli, Mudd and Niemann, 1952), cause foetal abortion and uterine haemorrhage, and elicit the reaction known as the Schwartzman phenomenon (Takeda and Tsuchiya, 1953).

A study of the literature established that strains of *E. coli* contained endotoxins with fairly clearly defined properties, but failed to show whether the endotoxins were confined to pathogenic strains. Following the work of Smith (1927) and Smith and Little (1927), who found that broth cultures of strains of *E. coli* obtained directly from the ileum of scouring calves were highly toxic for cows and calves when given intravenously, Lovell observed similar symptoms in calves injected with an antigen prepared by Boivin's method from a strain of *E. coli* pathogenic for calves (Lovell, unpublished).

The present investigation was undertaken, using strains of *E. coli* derived from calves, to determine whether the toxic components of organisms responsible for calf scours differed in any major chemical or biological properties from those not apparently associated with disease, and whether the virulence of the calf-pathogenic strains could be directly referred to their toxins.

As it has not been possible to work with fractions as highly purified as Westphal's lipopolysaccharides, our aim has been to extract all the toxic material present in the bacteria, by using modifications of Boivin's technique for the isolation of *antigène complet.*

Since the various compounds prepared by us and described in this paper are closely allied to the *antigène complet* they are described simply as antigens and not as the more specific and purer lipopolysaccharides.

METHODS

Selection of Strains

Six strains of *E. coli* known to be associated with outbreaks of white scours in calves were selected from a large group available (Wood, 1955;

TABLE 1

COMPARATIVE PROPERTIES OF TWELVE STRAINS OF *E. COLI* OF CALF ORIGIN

Properties common to group	*No.*	*Strain No. RVC*	*Antigenic structure*	*Reference*	*Description of individual strains*
Pathogenic, used as typing strains. Commonly invasive. Virulent. All strains smooth. Biochemical tests show that all belong to Faecal Type 1	2	330	078:K80B	Ingram *et al.* (1953)	Virulent (i.e. very invasive). Strain of frequent occurrence in septicaemia and calf scours.
	4	1787	0137:K79L	Rees (1957)	As 330, these are the most virulent strains frequently associated with septicaemia.
	5	608	unknown	Wood (1955)	Associated more frequently with local intestinal infection than with septicaemia.
	6	1034	unknown	Wood (unpub.); see Rees (1958)	Similar to 608.
	8	118	09 KA?	Ingram *et al.* (1953), Rees (1958)	Similar to 608.
	12	101	035 K?	Ingram *et al.* (1953)	Virulent (invasive) strain. Similar to 330).
Presumably non-pathogenic strains for calves. Apparently non-invasive non-virulent. All strains apparently smooth. All belong to Faecal Type 1	1	1698	unknown	—	Isolated (1955) from a mixed culture of ileal contents of apparently normal calf, 21 days.
	3	2006	unknown	—	Isolated (1955) from intestinal contents of apparently normal calf 21 days.
	7	1398	unknown	—	As for 2006 (1954)
	9	1385	unknown	—	As for 2006 (1954)
	10	1400	unknown	—	As for 2006 (1954)
	11	1396	unknown	—	As for 1698

Rees, 1958). For comparison six other strains were selected at random from a number isolated from the intestinal contents of apparently healthy animals. The antigenic properties of most of the first group have already been studied (Wood, 1955; Rees, 1958). Some properties of the twelve strains are included in Table 1.

Cultivation of Organisms

For each strain four trays of nutrient agar enriched with 0·1 per cent glucose (pH 7·6) were used. Each tray was covered with greaseproof paper and autoclaved before seeding with a 24 hour broth culture of the appropriate organism. After 18 to 24 hours at 37 to 40° the bacteria were harvested by means of a glass scraper into a ball mill using about 100 ml. of water to aid transfer. After homogenising for 20 to 30 minutes the volume was noted and aliquots removed for preparation of acetone-dried material and for dry weight determination. The remainder was worked up for antigen as described below.

TABLE 2

NOTES ON PREPARATION OF ANTIGENS FROM TWELVE STRAINS OF *E. COLI*

Strain No.	*Pathogenic strains*	*Strain No.*	*Non-pathogenic strains*
RVC330	As set out in Fig. 1.	RVC1698	An equal volume of ether was added to the alcohol in the final stage to precipitate the antigen. This formed a tarry material. On redissolving in water and reprecipitating with alcohol this formed a whitish powder.
1787	Ethanol insufficient to precipitate the antigen; equal vols. of ethanol and ether precipitated the product as a brown-black coherent mass. On redissolving in water, centrifuging to remove débris, and reprecipitating with an equal volume of ether/ethanol (50/50), repeating the process, the material was obtained finally as a white friable powder.	,, 2006 ,, 1398 ,, 1385	As for 330 As for 330 As for 330. A little ether was necessary to aid final precipitation of the antigen.
,, 608	As for 330.	,, 1400	An equal volume of ethanol was added to the bacterial suspension before adding the trichloracetic acid, because the latter alone failed to precipitate the protein.
,, 1034	This strain was very mucoid. Since trichloracetic acid failed to precipitate the protein, the acid was removed by dialysis against tap water (24 hrs. 15°C) and the residual suspension treated with an equal volume of ice cold acetone. The precipitated proteins were removed by centrifuging and the supernatant worked up in the usual way.	,, 1396	As for 1400.
,, 118	As for 1034		
,, 101	As for 1034		

Preparation of the Antigens

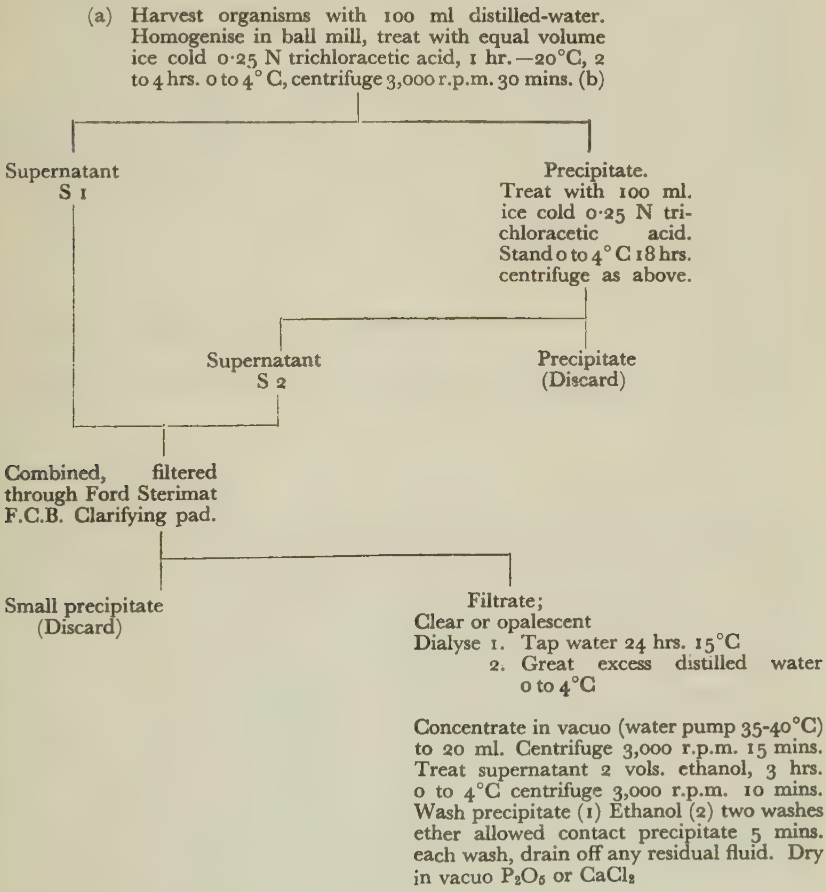
The problem was approached from two angles. First, attempts were made to extract the antigen from acetone-dried bacteria (*E. coli* RVC330) by a variety of techniques (Table 2) and secondly, an extraction technique based on Boivin's method was developed for freshly harvested organisms.

Methods of treatment of dried bacteria before extraction are summarised below.

Tryptic digestion (Raistrick and Topley, 1934). Dried bacteria (2·44 g.) were ground up with commercial trypsin (0·5 g.), tap water (100 ml.), and toluene (5 ml.), the pH adjusted to 8·5 with aqueous sodium carbonate and the mixture kept at 37 to 40°C. On the second day more trypsin (0·1 g.) was added. After 5 days the solution was neutralised (pH 7·0, dilute hydrochloric acid), concentrated to 20 ml. in vacuo (40 to 50°C, water pump), clarified by filtration and centrifugation and worked up as described below.

Fig. 1.

Flow sheet summarising final process, modified Boivin technique, for the preparation of antigens from strains of *E. coli.*



(a) Harvest organisms with 100 ml distilled-water. Homogenise in ball mill, treat with equal volume ice cold 0·25 N trichloracetic acid, 1 hr. —20°C, 2 to 4 hrs. 0 to 4° C, centrifuge 3,000 r.p.m. 30 mins. (b)

Supernatant S 1

Precipitate. Treat with 100 ml. ice cold 0·25 N trichloracetic acid. Stand 0 to 4° C 18 hrs. centrifuge as above.

Supernatant S 2

Precipitate (Discard)

Combined, filtered through Ford Sterimat F.C.B. Clarifying pad.

Small precipitate (Discard)

Filtrate; Clear or opalescent
Dialyse 1. Tap water 24 hrs. 15°C
2. Great excess distilled water 0 to 4°C

Concentrate in vacuo (water pump 35-40°C) to 20 ml. Centrifuge 3,000 r.p.m. 15 mins. Treat supernatant 2 vols. ethanol, 3 hrs. 0 to 4°C centrifuge 3,000 r.p.m. 10 mins. Wash precipitate (1) Ethanol (2) two washes ether allowed contact precipitate 5 mins. each wash, drain off any residual fluid. Dry in vacuo P_2O_5 or $CaCl_2$

Notes

(a) This refers to the growth of organisms from one large tray (see text)

(b) Except for minor variations such as described in the text when precipitation with trichloracetic acid alone is unsatisfactory.

Trichloracetic acid (Boivin, and Mesrobeaunu, 1933). Dried bacteria (2·73 g.) were extracted with trichloracetic acid (0·25 N) as described below and as illustrated in Fig. 1.

Diethylene glycol (Morgan and Partridge, 1940). Dried bacteria (2·75 g). were ground up in diethylene glycol (30 ml.) and toluene (10 ml.), and kept at 37 to 40°C. The mixture was stirred for 3 hours, allowed to remain for a further 18 hours, centrifuged at 3,000 r.p.m. for 30 minutes, dialysed against tap water and treated with trichloracetic acid as described below.

Saline. Dried bacteria (2·39 g.) were mixed with saline (sterile, 0·85 per cent w/v., 30 ml.) and toluene (10 ml.) and kept at 37 to 40°C for 18 hours. The suspension was cooled and the filtered fluid worked up as described below.

Treatment of the preliminary digests or extracts followed a scheme essentially as shown on the Flowsheet (Fig. 1). Because yields of the antigens were not very good attention was directed to an investigation of extraction of freshly harvested material. Since time of contact with trichloracetic acid appeared to be most important, this also was investigated (Table 3). Owing to the variation in the physical nature of the bacterial growth it was found necessary to make certain variations in the general technique. Thus, when growth was particularly mucoid, 0·25 N trichloracetic acid was unable to cause complete precipitation, and it was necessary to add alcohol or acetone to the mixture. The variations in the technique are shown in Table 4.

Chemical Estimations

Nitrogen. Essentially the microkjeldahl-Nessler technique described by Delory (1949).

Phosphorus. The method for organically bound phosphorus described by Kabat and Meyer (1948).

Reducing Sugar. The antigen was hydrolysed for 8·0 hours with hydrochloric acid (2 N). The digest was cooled, neutralised with sodium hydroxide (2 N) and the sugars determined by the method of Hagedorn and Jensen using glucose as a standard. Shorter periods of hydrolysis were less satisfactory for most of the strains investigated. These observations are in general agreement with those of Westphal *et al.* (1952).

Glucosamine. This was estimated in 8·0 hour acid hydrolysates by the Elson-Morgan reaction with acetylacetone and *p*-dimethyl-aminobenzaldehyde as described by Cramer (1954).

Chromatography. Only small quantities of antigens were available. Efforts were, therefore, concentrated on the identification of the major sugar components. Hydrolysis was effected by 3 per cent v/v mineral acid (nitric, hydrochloric or sulphuric). Hydrochloric and nitric acid were removed by concentration in vacuo (40 to 50°C, water pump) and sulphuric acid by treatment with hot aqueous barium hydroxide. On the whole the last method was the best and is described below in some detail. The antigen (10 mg.) was hydrolysed in a boiling water bath for 6·0 hours with sulphuric acid (5N, 5 ml.). Lipoid material separated out but was not removed until completion of hydrolysis. Hot aqueous barium hydroxide (10 per cent w/v) was added to the hot hydrolysate until the pH was brought to 5·5. The mixture was filtered through a Whatman

No. 41 paper and then through a little alumina. The precipitates and alumina were well washed with hot water till they gave little or no reaction with Molisch's reagent. The filtrate and washings were combined and evaporated to dryness in vacuo (30 to 40°C, water pump). The semi-crystalline residue was dissolved in water (0·10 to 0·15 ml.) for chromatography. Whatman No. 1 filter paper sheets were used for chromatographic analysis and 10 to 25 μl drops of the hydrolysate applied in the usual way. Occasionally dilution was necessary because very high concentrations of certain mixtures of sugars were difficult to separate, e.g. glucose and galactose (Fig. 3). Standard sugar solutions (1 per cent w/v) were applied in 5 μl drops. Several runs were made using hydrolysates resulting from the three acids and varying solvent systems. The best of these appeared to be (i) *n*-butanol-ethanol-water (4:1·1;1·9), (Hough, Jones, and Wadman, 1950); (ii) *n*-butanol-pyridine-water (3:2:1·5), (Luderitz and Westphal, 1952). This separates glucose and galactose. (iii) Collidine saturated with water (Partridge, 1948; Luderitz and Westphal, 1952).

Various developing agents were used, principally *p*-anisidine hydrochloride in *n*-butanol saturated with water (3 per cent w/v), aniline phthalate in water saturated with *n*-butanol and the Elson-Morgan reagent for sugar amines and acetylated sugar amines.

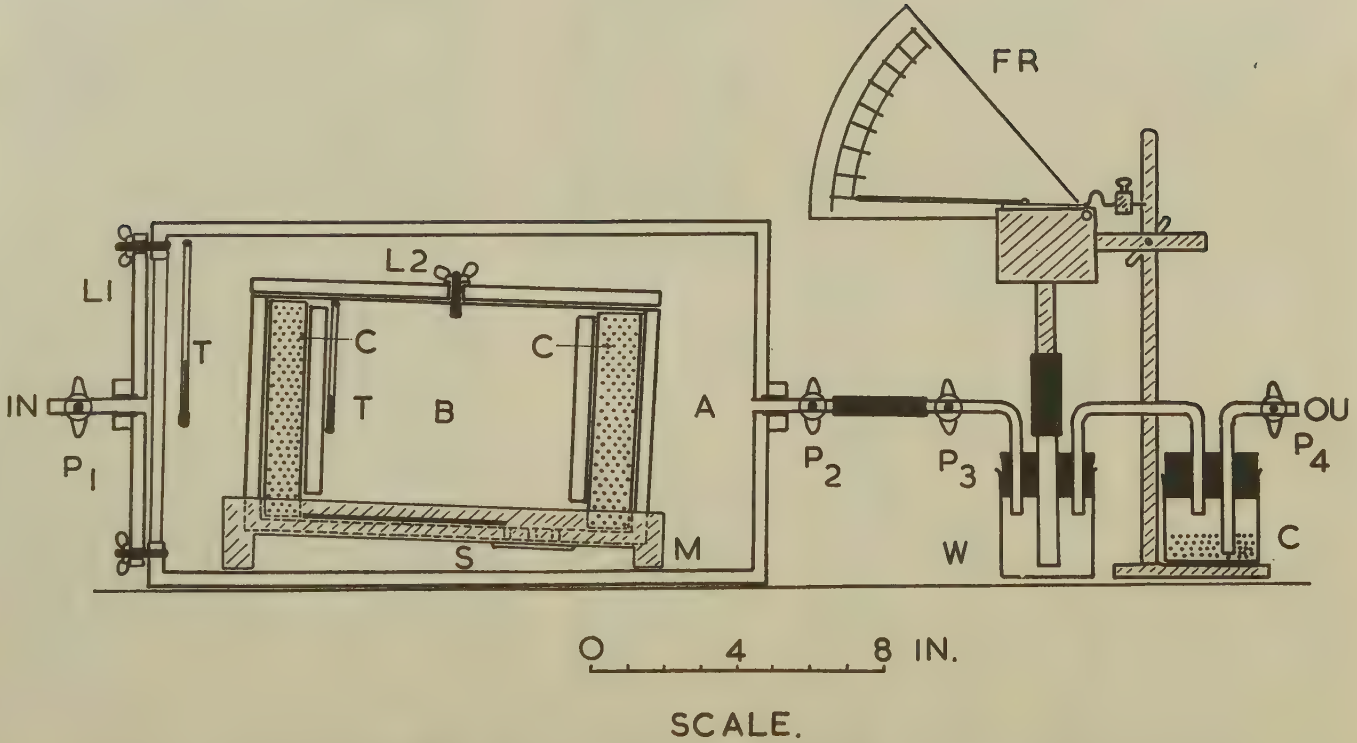
Toxicity.

Solutions of the antigen or suspensions of finely ground acetone-dried bacteria were sterilized at 56°C for 1·0 hour before administration. Mice were used for toxicity tests and were maintained on normal diets before, during and after experiments. Groups of four or more animals were used over a wide range of dose levels. The solutions or suspensions were administered by intravenous, intraperitoneal, intracranial and oral routes. Mortalities were recorded over 5-day periods following administration.

Oxygen Consumption

A limited number of determinations were made on rabbits. The apparatus used is shown in Fig. 2. It consisted of two Perspex chambers, an outer (A) fitted with a movable lid (L1) and an inner (B) for housing the animal. The inner chamber was placed at a slight angle on a metal tray (M) with drainage slits (S) to take away urine and faeces. It was fitted at each end with movable perforated zinc containers (C, C) containing Protosorb for absorbing CO_2. The outer chamber was fitted with inlet (P1) and outlet (P2) taps. The latter was connected to a floatmeter (FR) approximately 70 ml. capacity and calibrated in 10 ml. divisions. This was attached to another Protosorb tray (C). After the animal was placed in the inner chamber a slight positive pressure of air, or air mixed with O_2, was applied to raise the floatmeter to its maximum elevation. The time was then taken for the consumption of successive 10 ml. quantities of oxygen. This was repeated several times with ample aeration of the whole equipment (taps P1, P2, P3, P4 all open) and recharging (Harvey, 1958). Considerable attention was directed to the pre-treatment oxygen consumption levels in each case. This type of equipment was easy to assemble and to manipulate and has proved very suitable for this type of assay.

Fig. 2.


FR
L2
L1
T
C
C
T
B
A
IN
P1
P2
P3
P4
OU
W
C
S
M
0
4
8 IN.
SCALE.

Apparatus for measurement of oxygen consumption of rabbits. For lettering see text.

Body Temperatures

These were measured rectally on rabbits, again with particular attention to pre-treatment values.

Blood Sugar

Estimated by method of Hagedorn and Jensen.

Serological Experiments

Following the methods used by Lovell (1927), Wood (1955) and Rees (1958) for serological investigation of strains of *E. coli* of bovine origin, antisera were prepared in rabbits against the 12 strains of *E. coli*, and cross-agglutination tests were performed.

Agglutinating suspensions. K antigen suspensions were prepared from living suspensions of 0·1 per cent glucose-agar cultures, standardised to Brown's tube 1. O antigen suspensions were prepared by steaming dense suspensions, and adjusting to the same opacity, except for strain RVC118, in which 2 hours at 120°C was necessary to destroy the A-type capsule. (Wood, 1955). O and K antibody titres were determined by tube agglutination tests.

Some preciptin tests were carried out by the method of Lovell (1937) using Dryerre tubes with appropriate concentrations of homologous or heterologous antiserum, and doubling dilutions of the antigen solutions.

RESULTS

Tests for Significance

The comparison of equal or unequal groups of results by a modification of the "t" (Student) Test was made according to the formula given by Brownlee (1949), and the appropriate values of t ($P = 0{\cdot}02$) are given in the tables of results.

Preparation and Chemical Composition of the Antigens

From the evidence summarized in Tables 3, 4 and 5 it is clear that extraction of freshly harvested organisms results in the best yields of the Boivin type antigens. Preliminary precipitation and drying by acetone renders the bacteria very resistant to all forms of extraction although the products obtained have similar if not identical chemical composition and levels of toxicity. The main chemical differences between the products of the "dry" (process A) and "wet" (process B) methods appear to be high concentrations of nitrogen and reducing sugars and lower concentrations of phosphorus in the former. It is probable that the use of acetone in process A accounts for this since it would dissolve greater quantities of phospholipid than would aqueous trichloracetic acid alone. Comparison of the pathogenic and non-pathogenic strains reveals no statistically significant differences between the major chemical components (Table 5; $t < 2{\cdot}26$ or $2{\cdot}23$).

The exact nature of the protein moiety of the lipopolysaccharide has not been determined but in view of its relative solubility it may well have a fairly low molecular weight. The lipoid from the

TABLE 3

EFFICIENCY OF SUCCESSIVE EXTRACTIONS OF *E. COLI* RVC330 WITH 0·25 N TRICHLORACETIC ACID AT 0·4°C

Extract No.	*Contact with 0·25 N trichloracetic acid (hrs.)*	*Antigen*			*Nitrogen g./100 g. antigen*	*Phosphorus g./100 g. antigen*	*Nitrogen phosphorus ratio*	*Approx. LD 50 mg antigen per mouse*
		mg.	*per cent of total*	*g./100 g. bacteria*				
1.	3	340·0	42·6 } 81·3	3·24	6·6	2·0	3·3	>0·02 <0·10
2.	22	308·3	38·7 }	2·96	6·4	2·2	3·0	>0·02 <0·10
3.	27	67·7	8·5	0·64	5·5	3·1	1·8	>0·02 <0·10
4.	69	40·4	5·1	0·38	2·5	0·6	4·5	>0·10 <0·50
5.	102	32·6	4·1	0·31	4·2	0·7	6·5	>0·5 <2·5
6.	142	8·7	1·1	0·08	3·6	2·7	1·7	>0·5
Totals	—	797·7 mg. from 10·52 g. bacteria	100·0	7·93	—	—	—	—

TABLE 4

COMPARISON OF PROPERTIES OF THE ANTIGEN FROM *E. COLI* RVC330 PREPARED BY DIFFERENT METHODS

Type of extraction	*Acetone dried bacteria extracted (g.)*	*Properties of antigen*					
		Yield		*Phosphorus g./100 g. antigen*	*Nitrogen g./100 g. antigen*	*Reducing sugar g./100 g. antigen*	*Approx. LD 50 mg./mouse*
		wt. mg.	*g./100 g. bacteria*				
1. Trypsin digestion	2·44	41·5	1·7	0·4	5·0	57·0	<0·1 >0·05
2. Boivin trichloracetic	2·73	98·8	3·6	0·1	10·3	57·0	<0·025 >0·01
3. Diethylene glycol	2·75	57·1	2·1	0·5	7·0	51·0	<0·1
4. Saline: trichloracetic	2·39	18·9	0·8	0·4	10·4	31·2	<2·5 >1·0

TABLE 5

COMPARISON OF CHEMICAL PROPERTIES OF ANTIGENS PREPARED FROM TWELVE STRAINS

	Yield antigen g./100 g. bacteria		*Nitrogen g./100 g. antigen*		*Phosphorus g./100 g. antigen*		*Reducing sugar g./100 g. antigen*		*Nitrogen phosphorus*		*Reducing sugar nitrogen*		*Reducing sugar phosphorus*	
Process (b)	*A (a)*	*B (a)*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Six Pathogenic strains	1·3±0·6	6·2±4·7	5·2±0·6	4·2±1·9	0·41±0·15	1·37±0·42	57·0±13·9	35·0±6·4	16·7±13·7	3·4±2·8	11·0±3·0	10·1±4·2	171·0±106·0	20·4±6·4
Ratio between processes (A = 1)	4·8		0·8		3·3		0·6		0·2		0·9		1·2	
Six non-pathogenic strains	1·4±0·5	7·8±5·3	4·1±1·4	3·3±1·0	0·28±0·27	1·54±0·36	49·4±8·0	35·1±3·5	28·3±18·9	2·3±1·1	11·5±2·7	11·3±2·5	338·0±263·0	23·7±6·6
Ratio between processes (A = 1)	5·5		0·8		5·5		0·7		0·08		1·0		0·07	
t Pathogenic—non-pathogenic strains Found:—(c)	0·31	0·53	1·6	1·01	0·46	0·68	0·92	0·25	1·23	0·91	0·21	0·60	1·47	0·90
Theory:—	2·26	2·26	2·26	2·26	2·26	2·23	2·26	2·23	2·26	2·23	2·26	2·23	2·26	2·23

Notes.

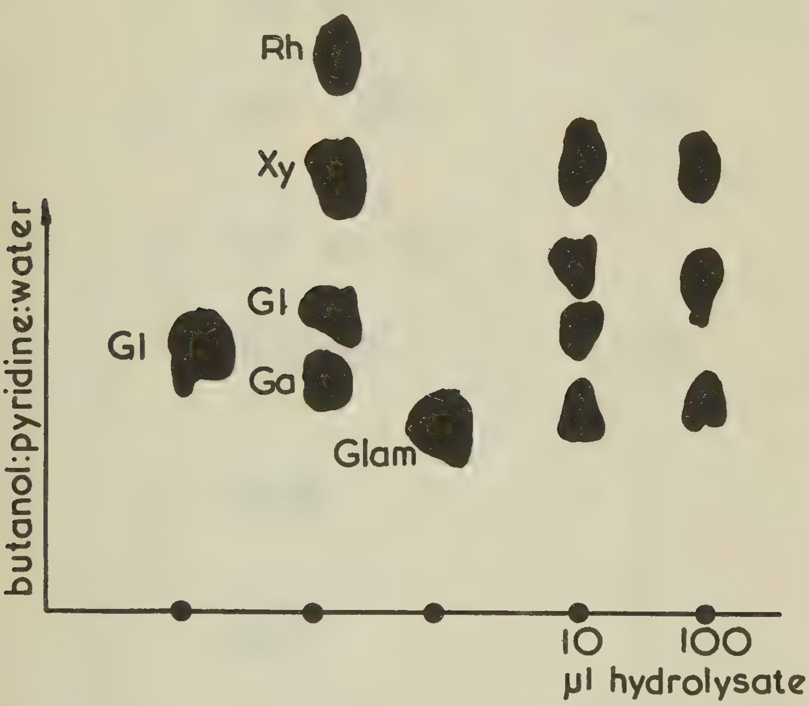
(a) Means of 5 or 6 samples ±SD.. The theoretical value of *t* for significance at a 5 per cent level (P = 0·02) is either 2·26 for 10 degrees of freedom (12 samples), or 2·23 for 9 degrees of freedom (11 samples).

(b) Process A trichloracetic acid extraction of acetone dried material, Process B trichloracetic acid extraction on freshly harvested bacteria (See Fig. 1).

(c) No significant differences.

antigen of *E. coli* RVC330 appeared to contain no steam-volatile fatty acids and this agrees with the observations of Westphal and Luderitz (1954) on the composition of lipoids from various Gram-negative organisms.

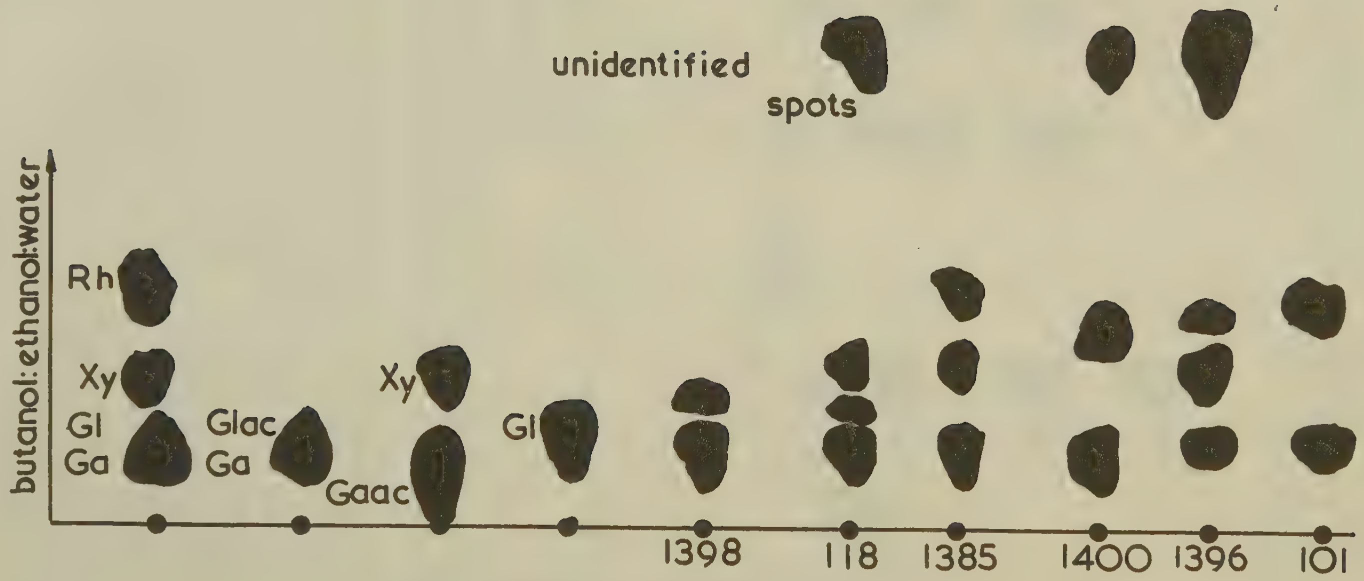
Fig. 3.



Descending chromatogram of acid hydrolysate of antigen from *E. coli* RVC330. Rh=Rhamnose, Xy=Xylose, Gl=Glucose, Ga=Galactose, Glam=Glucosamine. Note that overloading of spots, e.g. 100, µl. may prevent separation of some sugars.

Repeated chromatographic analyses revealed the fairly constant presence of glucose, galactose, xylose and glucosamine. Rhamnose was identified in all strains except 1034. Uronic acids, and acetylated sugar amines were noted infrequently (Table 6). Occasionally fast running 'spots' were observed (Fig. 4.) These gave fairly typical colour reactions with *p*-anisidine or aniline phthalate. Although insufficient material was available to make a positive identification it is possible that these 'spots' were occupied by some member of a newly identified family of sugars, the deoxymethylpentoses recently described (Davies, Staub, Fromme, Ludertiz and Westphal, 1958). Purer antigenic polysaccharides are required in greater quantities to permit definite identification of these interesting and biologically significant compounds.

Fig. 4.



Ascending chromatogram of nitric acid hydrolysates of antigens from several strains of *E. coli*. Initials as in Fig 3 with Glac=Glucuronic Acid, Gaac=Galacturonic Acid.

TABLE 6

CHROMATOGRAPHIC IDENTIFICATION OF SUGARS FROM ACID HYDROLYSIS OF ANTIGENS FROM TWELVE STRAINS

Strain	*Constituent identified*						
	Galactose + Glucose	*Galactose*	*Glucose*	*Xylose*	*Rhamnose*	*Glucosamine*	*Unidentified fast running 'spots'*
330	+	+	+	+	+	+	+
1787	+	+	+	+	+	+	—
608	+	+	+	+	+	+	—
1034(c)	+	—	—	+	—	+	—
118	+	+	+	+	+	+	+
101	+	+	+	+	+	+	—
Strains in which sugar identified	6/6	5/6	5/6	6/6	5/6	6/6	2/6
1698	+	+	+	+	+	+	—
2006	+	+	+	+	+	+	—
1398	+	+	+	+	+	+	—
1385(a)	+	+	+	+	+	+	—
1400	+	+	+	+	+	+	+
1396(b)	+	+	+	+	+	+	+
Strains in which sugar identified	6/6	6/6	6/6	6/6	6/6	6/6	2/6

Notes on additional 'positive' identifications.
(a) Glucuronic acid in 1385, 1396
(b) Galacturonic acid in 118, 1385
(c) Acetylglucosamine in 1034

Toxicity

Administration of antigens from all strains by any of the routes resulted in similar toxic effects in all animals. The products demonstrated a fairly wide range of toxicity to mice; in general the intraperitoneal route of administration was more effective than intravenous or intracranial (Tables 3, 4 and 7).

The LD 50 values of pathogenic strains ranged from 0·02 to 2·5 mg. antigen for mice and for the non-pathogenic strains from 0·02 to 1·0. As far as can be ascertained there is no significant difference between the two groups of substances and the variations can probably be attributed to differences in purity of the substances.

Massive doses of acetone-dried bacteria or of suspensions of the antigen (*E. coli*, RVC330, RVC1698) administered by stomach tube to mice failed to produce any obvious signs or symptoms of intoxication and no 'scouring' was observed. Further, this condition was the exception rather than the rule. Administration of massive doses by other routes usually resulted in death before any blood could be detected in the faeces.

TABLE 7

COMPARATIVE TOXICITIES OF ANTIGENS PREPARED FROM THIRTEEN STRAINS (TWELVE CALF, ONE BABY). MG/MOUSE (INTRAPERITONEAL ROUTE)

	Pathogenic strains (Calves)						*Baby strain*	*Non-pathogenic strains (calves)*					
Material	*330*	*1737*	*608*	*1034*	*108*	*101*	*0 111*	*1698*	*2006*	*1398*	*1385*	*1400*	*1396*
Antigens	0·10	0·02	0·10	<2·50 >0·50	2·50	0·10	0·02	0·02	0·02	<0·50 >0·10	<0·10 >0·02	<0·50 >0·10	<0·50 >0·10
Dried bacteria	<0·20	<0·20	<0·20	<0·20	<5·0 >1·0	<1·0 >0·20	1·0	0·2	<5·0 >1·0	<0·20	1·00	0·20	<1·0 >0·20

Payne and Rhee (personal communication) have reported that 1 mg. of antigen from *E. coli* RVC330 caused placental haemorrhage and foetal abortion in 13 day pregnant rats. In order to investigate the scouring effect still further Dr. Joan Taylor carried out assays with massive doses of antigens and with the live parent strains on isolated rabbit intestine (De, Bhattachyra and Sarkar, 1956). The results of these experiments were as follows:

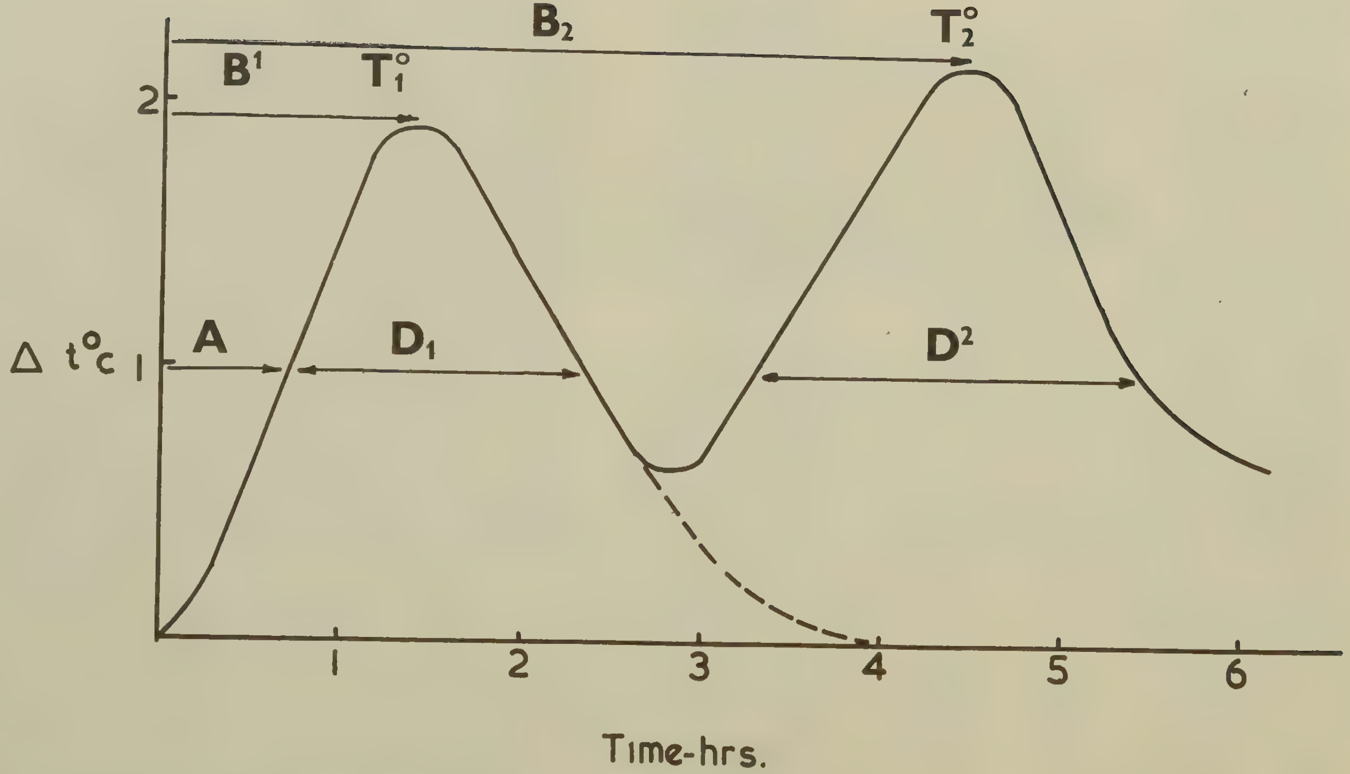
	E. coli		*Effect*
Pathogenic strain:	RVC330	Culture	Dilatation of gut, mucus, blood, etc.
	,, Antigen		No dilatation of gut
Non-Pathogenic Strain:	RVC1398	Culture	No dilatation of gut
	,, Antigen		No dilatation of gut

Severe symptoms of poisoning in mice by antigens from all strains of *E. coli* included lethargy and uncontrolled micturition. Body temperatures of mice were not taken but animals severely poisoned felt cold to the touch. No obviously toxic material other than the antigen could be identified and it is reasonable to assume that all the toxicity of dried samples of the twelve strains of *E. coli* can be accounted for by the antigen.

Other Physiological Tests

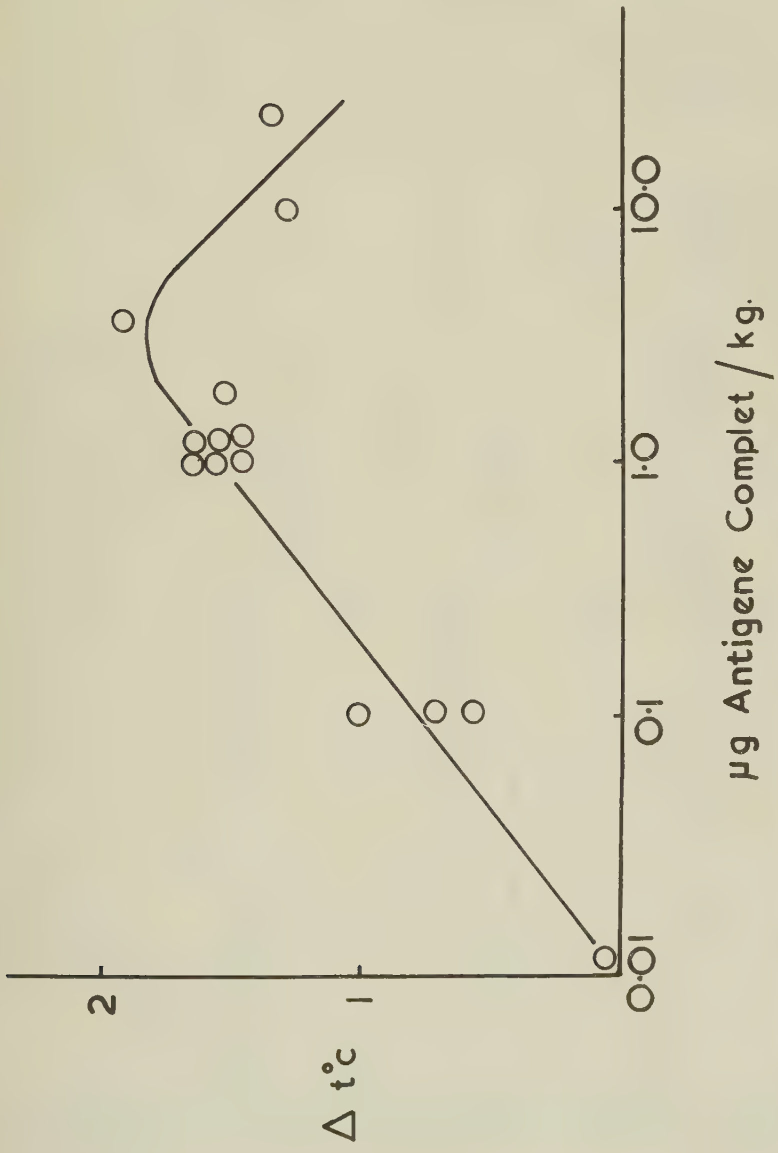
One of the most marked biological activities of all Gram negative antigens or lipopolysaccharides is their pyrogenicity in very small doses (<0·1 μg/kg.). Several observers (Grant, 1950; Grant and Whalen, 1953) have noted a double 'peak' temperature effect with increasing doses of bacterial pyrogens in man and in rabbits. This has been noted in some of the strains examined but it was not particularly marked. In order to ascertain the lowest dose level to give the maximum effect, a dose response curve was made for the antigen from *E. coli* RVC330 (Fig. 6). This followed a similar pattern to that described by Westphal *et al.* (1952) for a purer and more active lipopolysaccharide. Antigens from all strains except one—RVC118—resulted in significant pyrogenic activity in rabbits. It proved particularly difficult to isolate the antigen from this strain and it is believed that some activity may have been destroyed in the course of preparation. A stylised double peak response is given in Fig. 5 to explain more fully the details of the effects of the individual pyrogens at a single dose level, as given in Table 8. No statistically significant differences were noted between pyrogenic responses of antigens produced by pathogenic and non-pathogenic strains respectively. Oxygen consumption was stimulated by a single 20 μg dose of *E. coli* RVC330 administered intravenously

Fig. 5.



Stylised diagram of double peak effect of a single dose of a bacterial pyrogen.

Fig. 6.



Dose response of the pyrogenic effect on rabbits of antigen from *E. coli* RVC330. Intravenous injection.

and the peak value appeared slightly in advance chronologically of the first temperature maximum (Fig. 7). There was no second peak. Blood sugar levels, certainly at the dose investigated, were unaffected to any extent. Possibly at higher or slightly sub-lethal levels hyper- and hypoglycaemia might result (Delafield, 1934).

Following an unpublished observation by one of our colleagues, tests were carried out to investigate any histamine-like action of the

TABLE 8

PYROGENIC EFFECT ON RABBITS OF ANTIGENS PREPARED FROM TWELVE STRAINS. EACH ANIMAL GIVEN 1 μG/KG. INTRAVENOUSLY

		Peak 1 (a)				Peak 2 (b)		
	Strain	Latent period hr. (A)	First max. temp. increase T °C (T1)	Time taken to reach first max. temp. hr. (B1)	Duration of first temp. increase above 1°C. hr. (D1)	Duration of second temp. increase above 1°C. hr. (D2)	Second max. temp. increase T °C (T2)	Time taken to reach second max. temp. hr. (B2)
Pathogenic	330	0·5	1·6	1·0	2·0	—	—	—
	1787	0·5	1·5	0·6, 1·4	1·5	—	(0·7) (c)	(0·4)
	608	0·7	1·5	1·3	1·0	2·0	2·0	4·2
	1034	0·5	1·7	1·2	1·3	—	(0·7)	(3·7)
	118		no temperature increases noted.					
	101	0·8	1·2	1·0	3·2		2·0	2·7
	Mean ± SD	0·6 ± 0·14	1·5 ± 0·18	1·08 ± 0·28	1·45 ± 0·41	—	—	—
Non-Pathogenic	1698	0·6	1·4	0·8	1·0	3·5	2·2	3·5
	2006 (i)	0·5	1·7	1·3	1·0		2·7	—
	2006 (ii)	0·7	—	—	4·8	—	—	—
	1398	0·6	1·6	0·8	1·3	—	—	—
	1385	0·7	1·7	1·0	0·7		2·2	4·0
	1400	0·8	1·6	1·3	4·5		—	—
	1396	0·7	1·9	1·5	3·5			
	Mean ± SD	0·60 ± 0·09	1·65 ± 0·16	1·12 ± 0·29	1·00 ± 0·24		—	—
	t Pathogenic: non-pathogenic strains Found:—	(d) 0·08	1·4	0·23	0·24			
	Theory:—	2·26	2·26	2·23	2·45			

Notes.

(a) Capital letters A, T1 B1 etc. are shown in stylised response shown on Fig. 5
(b) Insufficient figures for evaluation of *t*.
(c) Figures in brackets indicate that these figures are of doubtful biological significance.

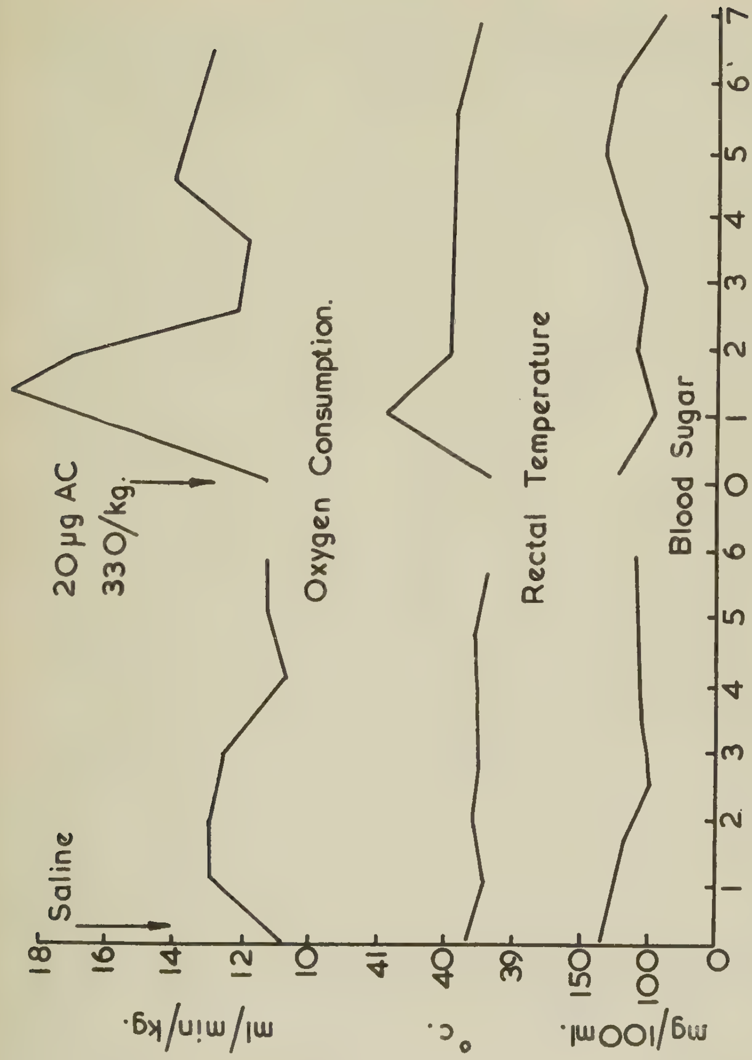


Fig. 7. Effects of a single dose (20 µg/kg.) of antigen from *E. coli* RVC330 on rectal temperature, oxygen consumption and blood sugar of a rabbit.

antigen from *E. coli* RVC330 on isolated guinea pig ileum. No such activity was demonstrated even though massive doses of the antigen were employed.

Serological Tests

Agglutination test. The results of cross-agglutination tests on 11 of the 12 *E. coli* strains are shown in Table 9. Strain RVC2006 which became rough during the experiment is excluded. Except for one unilateral heterologous reaction, the 11 strains are shown to be serologically distinct.

TABLE 9

CROSS-AGGLUTINATION TESTS ON ELEVEN STRAINS OF BOVINE ORIGIN

Anti-serum		*Antigen*										
		330	*1787*	*608*	*1034*	*118*	*101*	*1698*	*1398*	*1395*	*1400*	*1396*(a)
330	O	1024	—	—	—	—	—	—	—	—	—	—
	K	80	—	—	—	—	—	—	—	—	—	—
1787	O	—	2560	—	—	—	—	—	—	—	—	—
	K	—	640	—	—	—	—	—	—	—	—	—
608	O	—	—	5120	—	—	—	—	—	—	—	—
	K	—	—	160	—	—	—	—	—	tr 40	—	—
634	O	—	—	—	2560	—	—	—	—	5120	—	—
	K	—	—	—	80	—	—	—	—	—	—	—
118	O	—	—	—	—	2560	—	—	—	—	—	—
	K	—	—	—	—	40	—	—	—	—	—	—
101	O	—	—	—	—	—	1280	—	—	tr 320	—	—
	K	—	—	—	—	—	160	—	—	—	40	—
1698	O	—	tr 40	—	—	—	—	5120	—	—	—	—
	K	—	—	—	—	—	—	640	—	—	—	—
1398	O	—	—	—	—	—	—	—	20480	80	—	—
	K	—	—	—	—	—	tr 320	—	1280	—	80	tr 160
1385	O	—	—	—	—	—	—	—	—	5120	—	tr 160
	K	—	tr 40	—	—	—	—	—	—	1280	160	tr 32
1400	O	—	—	—	—	—	—	—	—	tr 320	2560	—
	K	—	—	—	—	—	—	—	—	—	1280	—
1396	O	—	—	—	—	—	—	—	—	tr 320	—	5120
	K	—	—	—	—	—	—	—	—	—	40	320

Note: (a) One of the strains, *E. coli* RVC2006, became rough during the experiment so it was excluded from the cross agglutination tests.

Precipitin tests. Positive precipitin tests with homologous (OK) antiserum were demonstrated by Dr. Rees with extracts from RVC330 prepared in 4 different ways. The strongest reactions were obtained with the tryptic and Boivin extracts (1·4 μg after 18 hours), and the weakest with the diethylene glycol extract (125 μg after 18 hours). The reaction developed more slowly with the Boivin extract than with the tryptic extract. Eight μg of antigen prepared from strains RVC1698, 330 and 608, and 4 μg of AC from RVC1034 were the lowest amounts of the antigen which gave positive precipitin tests with the corresponding antibacterial sera. No heterologous reactions were shown.

DISCUSSION

By using a modified version of the original Boivin technique, products conforming to the criteria for *antigène complet* (Boivin and Mesrobeaunu, 1935, 1936b) and typical of bacterial endotoxins were isolated from all the twelve strains of *E. coli* studied. Simple techniques confirmed the chemical and histological similarity of these products which structurally represent a very interesting group. Due to the large molecular size and limited solubility of the larger aggregates precise characterisation is difficult. It is not clear whether the antigen represents several allied toxic substances or is a single complex with several properties.

Our products comprised about 8 per cent of the dry weight of the bacterial cell, with a pyrogenic activity 50 times less than Westphal's highly purified pyrogen, which represented 1 per cent only of the dry weight (Westphal *et al.*, 1952). Braude, Carey, Sutherland and Zalesky (1955), using a Boivin method of extraction, found that endotoxin was equivalent to at least 22 per cent of the dry weight of *E. coli* cells, and cited the uniform distribution of Cr^{51} used to label the endotoxin as evidence of its homogeneity. The uniformity of the pyrogenic response of our products was a sensitive index of their physiological activity.

The few sugars consistently represented in hydrolysates of the Boivin extract are probably structural constituents of the somatic antigen. According to Webster, Landy and Freeman (1952) the K antigen of *E. coli* is a polymeric acid, possibly made up of *n*-acetylaminohexuronic acid units and containing only 2 per cent of reducing sugars. Although each antigen conformed to the specificity of the O antigen of the parent strain, some K may also have been present. Varying proportions of these two constituents may have accounted for slight variations in the chemical constitutions of different batches of the antigen. The presence of unidentified amino-compounds in addition to glucosamine may be evidence in favour of polypeptide components of the antigenic complex of *E. coli* (Kasai, 1952). Some of these amino-compounds may represent amino-acids of the cell wall, for example diaminopimelic acid (Work and Dewey, 1953).

In some strains the separation of lipopolysaccharides from proteins may be more difficult than in others. Webster, Sagin, Landy and Johnson (1955) considered that active lipopolysaccharide components of bacterial cells may exist in two types of structural unit, one free from protein and the other protein-linked. Ikawa *et al.* (1952) noted that fractions of *E. coli* lipopolysaccharides with similar toxicities and the same serological specificity have different molecular sizes.

As all the strains investigated had the same order of toxicity irrespective of their pathogenicity for calves it seems clear that the possession of endotoxin is not in itself sufficient to account for the pathological role of *E. coli* in calf scours. Boivin (1941) stated that intact antigenic and toxic structure were not alone sufficient for the high virulence of Salmonella, but that the capacity of the organisms to multiply in situ was affected by additional properties which were rapidly lost by cultivation on artificial media. Our observations on *E. coli* confirm Boivin's views and are in direct contradiction to the theory of Kauffmann (1954) that the virulence of enteropathogens varies as the toxicity of the organisms.

In *E. coli* virulence is apparently an attribute of the living organism. One of the possible causes of diarrhoea and scouring in calves may be the release of toxins by small numbers of invading bacteria. Irritation of the intestinal mucosa may also be set up by massive concentrations of locally dominant bacteria. Whereas adult animals are susceptible to injected toxins they have developed mechanisms, both specific and non-specific, to protect them against invading bacteria and their toxins. The young mammal, particularly if deprived of colostrum, is not so protected, so that organisms which are normal inhabitants of the alimentary canal may become harmful if conditions especially favour their multiplication. The relationship between host and parasite is a dynamic one, and the ability of certain strains to achieve dominance depends on host-parasite relations and external factors. With shifting equilibria the dominant strains may change and be replaced by others. Our observations suggest that all smooth strains of *E. coli* have similar toxic constituents and may be potentially virulent pathogens.

CONCLUSIONS

Three main conclusions may be drawn from the results of the experiments published in this paper.

Relatively simple analytical techniques have failed to reveal in the twelve strains of bovine *E. coli* any significant differences, or the presence of any specifically toxic product other than a lipopolysaccharide.

This substance is present in the virulent and non-virulent strains in concentrations that do not appear to bear any relation to virulence. On the basis of acute toxicity experiments on small animals, and a few on the isolated small instestine of rabbits no definite indications have been obtained that can explain the fundamentals concerning the mechanism of scouring. Quite clearly this property of virulent strains of *E. coli* is intimately associated with the viability of the organism, and not directly with the presence of a toxic lipopolysaccharide.

It is likely that similar findings and conclusions would have resulted from a survey of a much wider range of bovine coliform organisms. It is clear that the keys to the important problems of virulence and scouring in *E. coli* and other Gram negative organisms will be revealed only by more detailed studies of the essential structural materials, particularly the nucleic acids, of the bacterial cell.

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We wish to thank Dr. T. A. Rees for providing the strains of *E. coli* examined in this study, and for many helpful discussions. Prof. R. Lovell also gave much useful advice. Dr. Joan Taylor assayed some of our products by the intestinal loop method, and Mr. David Chessell gave a great deal of skilful technical assistance.

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FURTHER OBSERVATIONS ON BLOOD COMPONENTS IN LACTATING DAIRY CATTLE

By

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INTRODUCTION

Paterson (1957) and Horrocks and Paterson (1957) have reported the results of determination of leucocytes, 17-hydroxycortiocsteroids, glucose, ketone bodies and volatile fatty acids in the blood of dairy cattle in late pregnancy and early lactation. Plasma 17-hydroxycortiscosteroid levels increased one to two weeks before calving, as did the blood level of glucose. Both 17-hydroxycorticosteroid and glucose levels decreased immediately after parturition, and in many cases quite low levels of glucose were found for a short period postpartum.

The present paper reports a further study using a group of seven cows, all of which had had ketosis in previous lactations, and which were observed from about one week prepartum until about eight weeks postpartum. Two of these animals will not be considered, since one developed a severe staphylococcal mastitis and the other was inadvertently treated for suspected ketosis. Four animals remained normal throughout the period of study, and results from these confirm our earlier findings. One cow developed clinical ketosis and the results of observations and treatment will be described.

METHODS

The methods used were those described previously (Paterson, 1957; Horrocks and Paterson, 1957) with certain modifications.

Plasma 17-hydroxycorticosteroids. The use of a modified cell carrier for the Unicam spectrophotometer allowed readings to be made using only 1 ml. of reaction mixture in 1 cm. cells. The consequent decrease in volume of reagents increased the sensitivity of the method, the regression equation for the colour reaction being: 17 hydroxycorticosteroid (μg.) = 25.4 x optical density.

Leucocytes. Only eosinophils were counted, after 1:10 dilution of blood with a 0·2 per cent. (w/v) solution of eosin (yellow) in aqueous acetone (5 per cent. v/v acetone; Discombe's diluting fluid).

Ketone bodies. Whereas in previous work only acetone and acetoacetic acid were estimated, in the present work β hydroxybutyric acid was also estimated, using the direct method of Greenberg and Lester (1944).

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RESULTS

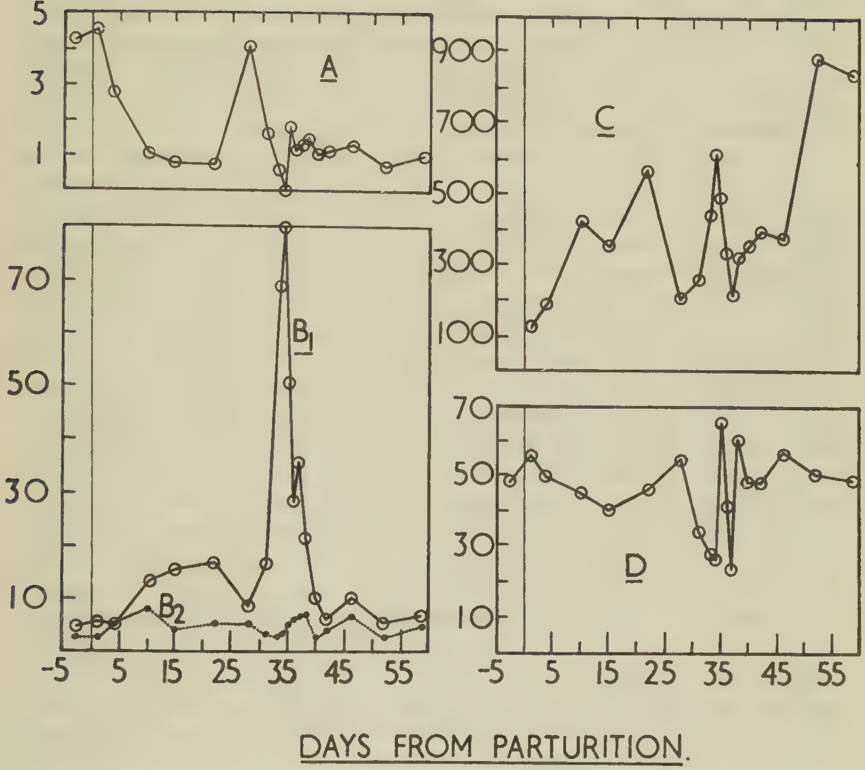
The results from the four normal animals are summarised in Table 1. The plasma levels of 17-hydroxycorticosteroids appear to

TABLE 1

BLOOD COMPONENTS IN NORMAL COWS IN EARLY LACTATION

		Prepartum 7 – 0 days	Postpartum (days)						
			0 – 7	8 – 14	15 – 21	22 – 28	29 – 35	36 – 42	43 – 56
Glucose mg./100 ml. blood	Mean ± S.D.	50·1 ± 7·7	47·6 ± 7·7	39·8 ± 2·4	39·9 ± 3·7	40·0 ± 5·3	44·2 ± 6·1	41·2 ± 5·0	44·4 ± 2·5
	Range	40 – 63	37 – 62	37 – 42	34 – 47	34 – 46	37 – 54	35 – 49	41 – 47
Total ketone bodies mg. acetone/100 ml. blood	Mean ± S.D.	3·9 ± 1·8	6·1 ± 3·4	13·2 ± 8·4	14·9 ± 13·3	14·0 ± 8·4	15·0 ± 10·9	15·5 ± 10·4	8·8 ± 2·6
	Range	1·2 – 6·5	2·9 – 15·1	1·6 – 23·4	4·2 – 40·1	7·1 – 28·4	4·2 – 34·9	7·9 – 35·8	4·6 – 12·4
Volatile fatty acids mg. acetate/100 ml. plasma	Mean ± S.D.	6·1 ± 3·5	4·4 ± 1·0	8·0 ± 5·3	6·6 ± 3·8	6·8 ± 2·8	3·1 ± 1·0	5·5 ± 2·4	3·0 ± 1·6
	Range	4·0 – 12·9	2·7 – 6·4	2·5 18·8	1·9 – 13·0	3·5 – 9·6	1·6 – 4·2	2·1 – 8·9	0·7 – 4·2
17-hydroxy-corticosteroids ug./100 ml.	Mean ± S.D.	2·7 ± 1·4	2·5 ± 1·9	1·8 ± 0·5	2·1 ± 1·0	1·8 ± 0·8	2·4 ± 0·8	1·3 ± 0·4	1·6 + 0·4
	Range	1·0 – 4·5	1·1 – 7·2	0·9 – 2·3	1.0 – 3·9	0·9 – 2·9	1·3 – 3·6	0·7 – 1·8	1·0 – 2·3
Eosinophils cells/ c.mm. blood	Mean ± S.D.	281 ± 225	164 ± 117	257 ± 240	342 ± 240	764 ± 457	480 ± 252	575 ± 175	889 ± 630
	Range	36 – 696	36 – 440	12 – 660	73 – 748	176 – 1441	209 – 836	297 – 825	110 – 1507

FIGURE I.



Blood components in a cow during the development of ketosis, and treatment with corticotrophin (160 I.U. administered on 34th and 37th days).
A. 17-hydroxycorticosteroids (μg./100 ml. plasma)
B^1. Total ketone bodies (mg./100 ml. blood)
B^2. Volatile fatty acids (mg./100 ml. plasma)
C. Eosinophils (cells/c.mm.)
D. Glucose (mg./100 ml. blood)

show no marked variation, but this is due largely to variation between animals. However, all four animals were similar, showing an increase in plasma 17-hydroxycorticosteroids just before or at parturition and while the level decreased soon after parturition, there was in all cases a later transient increase to about the level found at parturition (2·3 to 7·2 μg./100 ml. plasma). There was correlation between plasma corticosteroid levels and eosinophil levels, r = —0·31, by Fisher's z transformation of correlation coefficients for individual animals (0 to —0·67). One reason for the rather poor correlation is that the transient postpartum increase in corticosteroid was not always accompanied by an eosinopoenia.

In all four cases there was an increase in blood glucose just before parturition and a steady decrease to a lower level of 34 to 41 mg. per cent after parturition. The decrease in glucose levels between the first and second weeks postpartum is significant ($P = 0{\cdot}05$). Blood ketone body levels increased postpartum, the highest levels of ketone bodies coinciding with the lowest levels of glucose. Volatile fatty acid levels varied little, except that higher levels were found usually to accompany high levels of ketone bodies.

The results from the animal which developed ketosis are shown in Fig. 1. The blood changes shown by this animal until the 28th day postpartum were quite normal, and it appeared to be eating and milking normally. At the 31st day postpartum the animal refused concentrates and her milk yield had decreased. On the 33rd day postpartum the animal showed typical symptoms of ketosis including inappetence, decreased milk yield to at least 15 lb./day below normal and marked hypoglycaemia and ketonaemia. On the 34th day postpartum the condition of the animal was unchanged and corticotrophin 160 I.U. was given. This resulted in an increase in plasma 17-hydroxycorticosteroids, an eosinopoenia, a striking increase in blood glucose and a decrease in ketone bodies. On the 36th day there was improvement in the animal's condition and milk yield was increased by 10 lb. However, next day blood glucose had again decreased to a very low level and the ketone body level had increased, so a further 160 I.U. corticotrophin was given. This resulted in little change in plasma 17-hydroxycorticosteroids, a decrease in eosinophils, a marked increase in blood glucose and a decrease in the ketone body level. By the 39th day the animal was eating and milking normally and had apparently made a complete recovery. The animal was observed for a further three weeks and in that period was normal.

DISCUSSION

The results from one case of ketosis are presented for their interest, and not as a basis for discussion. The fact that a two year study involving twenty-nine cows, of which thirteen had had a history of ketosis in previous lactations, yielded only one case of ketosis for observation should not be allowed to detract from the value of this experimental approach. The incidence of clinical ketosis on the farms used in the period of study was very small; had there been available herds with a high incidence of ketosis, more success might reasonably have been expected. The apparent sudden onset of the disease in the case observed indicates that frequent blood sampling is desirable, say thrice weekly normally and at times of suspected abnormality, daily or twice daily.

The results from normal animals described in this paper confirm our earlier findings. Plasma 17-hydroxycorticosteroids increase in cattle just before calving and decrease after calving. Brush (1958) has obtained similar results but did not study his animals beyond one week after parturition. In the present work transient increases in plasma 17-hydroxycorticosteroids were seen in all four normal animals, and in seven of the twenty-two cases studied earlier. As stated in our earlier paper (Paterson, 1957) this transient postpartum increase does not appear to be related to the level of, or changes in, milk yield, and consequently was assumed not to be the result of a "lactation stress". It could be argued that stress might not be restricted to high yielding animals or animals at peak lactation.

Correlation between plasma 17-hydroxycorticosteroids and eosinophils was not very good, which confirms our earlier finding. There is considerable variation between animals, and this was also found in short term studies of animals to which corticotrophin had been given (unpublished observations). No reason can be offered, but it would appear to indicate that determinations of eosinophils alone at intervals such as those in the present work may not form a reliable basis for assessing adrenocortical function.

We have again found that in the normal animal rather low levels of blood glucose may be found in the early postpartum period. Sometimes the level of blood glucose is appreciably less than the accepted lower normal limit of 40 mg. glucose/100 ml. blood. In our earlier work (Horrocks and Paterson, 1957) there was no marked increase in ketone bodies coincident with this decrease in blood glucose, but at that time only acetone and acetoacetic acid were being estimated. In the present work β-hydroxybutyric acid was also estimated, and was found to form the major part of the ketone bodies. Further, it was found that there were considerable variations in the blood level of β-hydroxybutyric acid with little change in the levels of acetone and acetoacetic acid. Thus our finding in this paper that total ketone bodies increase markedly postpartum, particularly at times of lowered blood glucose levels, is not incompatible with our earlier finding that acetone plus acetoacetic acid varied little postpartum, since almost all of the variation in total ketone body levels is due to variation in the level of β hydroxybutyric acid.

Thus in the normal cow soon after parturition slight hypoglycaemia and ketonaemia may be seen, and while the average cow might show a glucose level of 39 mg. per cent. and a total ketone body level of 12 mg. per cent. (as acetone), more "abnormal" levels may be seen. In the present work two cows showed blood glucose levels of 34 mg. per cent. coinciding with total ketone body levels of 40 mg. per cent. Such observations suggest that either we must revise our concept of what are normal blood levels of glucose and ketone bodies, or that in normal animals at about 2 to 4 weeks after parturition there is a tendency to hypoglycaemia and ketonaemia which in some animals may be quite marked, although the blood levels of glucose and ketone bodies do not approach those seen in the clinically ketotic cow. When such "abnormal" values were seen in the present work this was the only apparent abnormality since the animals were eating and milking normally. It would be of interest to know the extent of such changes and their time-relationship to the onset of ketosis in ketotic animals.

CONCLUSIONS

Previous findings concerning certain blood components in normal lactating cattle have been confirmed and extended in a further study of 4 normal cattle.

The blood level of glucose, which is higher than normal at parturition, decreases significantly thereafter to quite low levels of about 35 to 40 mg. per cent.

In the present work β-hydroxybutyric acid was estimated in addition to acetone and acetoacetic acid. Ketone body levels show a transient increase postpartum which is largely in β-hydroxybutyric acid. This observation is not inconsistent with our earlier findings when only acetone and acetoacetic acid were measured. There was little variation in plasma volatile fatty acids.

Plasma levels of 17-hydroxycorticosteroids which are increased at parturition decrease thereafter, although there is a later transient increase. Blood eosinophil levels do not show good correlation with plasma 17-hydroxycorticosteroids.

Results from one case of ketosis are described.

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NEWCASTLE DISEASE IN THE GREY PARROT (*PSITTACUS ERITHACUS* L.)

By

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INTRODUCTION

The complete natural host range of Newcastle Disease virus is unknown. A scattered literature incriminates sixty or so species of birds as susceptible out of several thousand possibles, but from twenty or so species only has the virus been isolated and identified.

Four papers record Newcastle disease in birds belonging to the Order Psittaciformes and presumably to the Family Psittacidae. Farinas (1930) included unnamed species of parrots in his list of susceptible birds. Cooper (1932) discovered that Indian green parroquets (*Psittacula krameri* Scop.) were susceptible to parenteral administration of the virus. Malbrant (1942) noted, during an epizootic in French Equatorial Africa, that "Australian" parrots (species unnamed) and red-headed lovebirds (*Agaporius pullaria pullaria* L.) died. Zuydam (1952), in Holland, isolated Newcastle disease virus from the spleens of sick parroquets (*Psittacula krameri borcalis* Nearn) which had arrived from India. Conversely, Placidi and Santucci (1954) remarked that psittacine birds were not affected during an outbreak in a zoological garden.

The first major epizootic of Newcastle disease in Kenya for twenty years occurred in 1955 (Scott, Gibson and Danskin, 1956). Grey parrots (*Psittacus erithacus* L.) were among the species clinically affected. The history, clinical and post-mortem findings of the disease in the parrots and the characteristics of the isolated virus are herein recorded.

RESULTS

History

The grey parrots were trapped in the Belgian Congo and forwarded to Kampala in Uganda for sale as household pets. Nine parrots were sent on to a dealer in Nairobi, Kenya. Illness was noticed in the latter group shortly after arrival. The birds were removed from the dealer's premises for observation. None of his other stock, which included pigeons and budgerigars, exhibited signs of Newcastle disease, but two weeks later haemagglutination-inhibition (HI) antibodies were detected in the sera of two in-contact pigeons (*Columba livia*).

Clinical Findings

Eight parrots were affected and died; the remaining one was vaccinated with an inactivated vaccine. It remained healthy and did not develop HI antibodies. The disease first appeared in two

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TABLE 1

TEMPORAL OCCURRENCE OF NEWCASTLE DISEASE IN A GROUP OF PARROTS

Parrot number	*Days after the detection of the last case*	*Days after the detection of the first case*
1, 2	0	0
3	14	14
4, 5	12	26
6	15	41
7, 8	12	53

birds and thereafter spread slowly to the other six, the last two becoming ill 53 days after the first cases (Table 1). The duration of the illness was short and varied from one to three days. Respiratory symptoms were predominant and nasal and ocular discharges were profuse. The eyes were kept closed and the feathers were ruffled. Inappetance and general debility developed. Nervous symptoms were not observed.

Post-mortem Findings

The most noteworthy feature at post-mortem was the lack of significant changes. The carcases were in good condition but fevered. Four birds had a diffuse congestion of the proventriculus. Enteritis was marked and in one bird sloughing of the mucous membrane had occurred. Tracheitis was noted only once.

Isolation of Virus

Viral isolations were made from the spleens of four dead parrots. Spleen suspensions were prepared with nutrient broth containing antibiotics and inoculated into the allantoic cavity of fowl embryos, previously incubated for 10 days. When the embryos died 36 to 72 hours after inoculation, the allantoic fluids were harvested and tested for the presence of haemagglutinins by the Salk technique (Salk, 1944). The agents isolated contained haemagglutinins and were shown to be Newcastle disease virus by haemagglutination-inhibition tests using specific Newcastle disease antiserum. A typical result is recorded in Table 2. The original isolant was labelled Newcastle disease virus, Tiladam-Nairobi-1955 strain.

Characteristics of the Virus

The virus was twice passaged in fowl embryos and tests were carried out with infected allantoic fluids.

Pathogenicity for fowls. A 10^{-2} dilution of the virus was prepared in saline and 1.0 ml. was inoculated intramuscularly into 20 White

TABLE 2

HAEMAGGLUTINATION INHIBITION TESTS

Inhibition by known Newcastle disease immune serum of the haemagglutinins of the Tiladam-Nairobi-1955 strain of Newcastle disease virus which was isolated from grey parrots

Serum dilutions	*Viral dilutions*							
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
10^{-1}	x	x	x	x	x	x	x	x
10^{-2}	x	x	x	x	x	x	x	x
10^{-3}	o	o	o	⊕	x	x	x	x
10^{-4}	o	o	o	o	o	o	o	
Virus in negative serum	o	o	o	o	o	o	o	x
Serum alone	x	x	x	x				
RBC in saline	x	x	x	x				

x = no haemagglutinin
⊕ = 50 per cent haemagglutination
o = complete haemagglutination

Leghorn fowls. Respiratory and nervous symptoms were noticed on the third day after inoculation and deaths occurred on the fourth to the sixth day after inoculation. The fifty per cent death time was 4·7 days. Twenty fowls kept in close contact with 20 experimentally infected fowls died after exhibiting nervous and respiratory symptoms. The 50 per cent death time following contact infection was 11·5 days.

The virus was then titrated in White Leghorn fowls. Each tenfold dilution was inoculated into ten birds and the 50 per cent lethal dose, as calculated by the method of Reed and Muench (1938), was $10^{-6 \cdot 7}$ per ml. of allantoic fluid. The 50 per cent death time of the minimal lethal dose was 7 days.

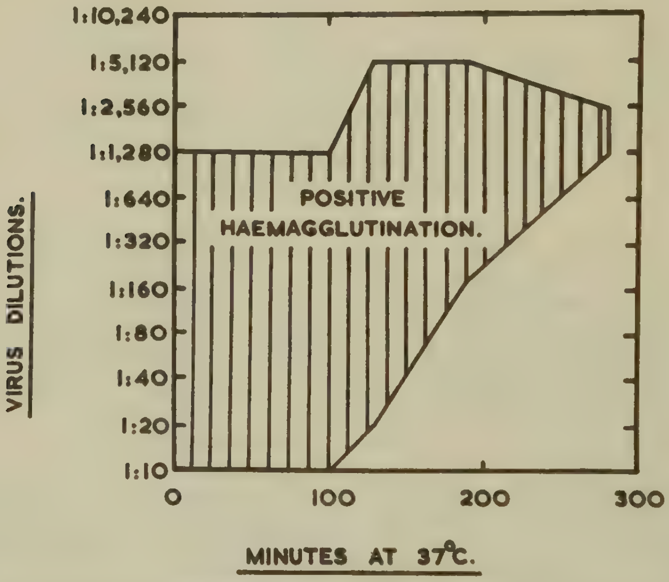
Pathogenicity for fowl embryos. The virus was similarly titrated in fowl embryos, using 10 embryos per dilution. The 50 per cent lethal dose was $10^{-9 \cdot 1}$ per ml. of allantoic fluid. The mean death time of the minimal lethal dose was 60 hours.

Erythrocyte spectrum of the haemagglutinin. The isolated viruses agglutinated erythrocytes from fowls, humans and guinea pigs. The erythrocytes of cattle, goats, hamsters and horses were not agglutinated.

Haemagglutination elution pattern. Serial twofold dilutions of virus and a one per cent suspension of fowl erythrocytes were used. The mixtures were shaken and allowed to settle on the bench at room temperature (23°C.). After 40 minutes the tubes showing agglutination were noted. The mixtures were again shaken, allowed to settle in a waterbath at 37°C. and examined for agglutination after 30 to 60 minutes. The procedure was repeated until five readings had been made. The resulting pattern (Fig. 1) indicated that the "parrot" virus was a medium elutor.

Heat stability of the haemagglutinin. Viral aliquots, diluted 1 in 5 with saline, were held in the 56°C. waterbath for 5, 10, 20 and 40 minutes and then transferred to ice-water. Serial twofold dilutions were prepared and one per cent fowl erythrocytes were added. Simultaneously, control aliquots held on the bench were likewise checked for haemagglutinins. The haemagglutinin survived exposure to 56°C. for five minutes but not for ten.

Fig. 1



Elution pattern of the Tiladam-Nairobi-1955 strain of Newcastle disease virus.

DISCUSSION

The strain of Newcastle disease virus isolated from parrots in Kenya was highly virulent for grey parrots which, once affected, rapidly succumbed. The same strain was also very virulent for fowls and fowl embryos. Hanson and Brandly (1955) classified strains of Newcastle disease virus according to their growth rate in fowl embryos which was measured by the mean death time following administration of the minimal lethal dose. Our strain would be typed by their classification as "mesogenic".

Despite the proven virulence of the strain, the spread of the disease through the parrots was slow. Either the invasiveness of the virus was low or the parrots possessed an innate resistance to contact infection. Innate resistance to Newcastle disease might explain the observation of Placidi and Santucci (1954) that psittacine birds escaped infection during an epizootic in a zoological garden whereas we and others have shown psittacine birds to be susceptible.

CONCLUSIONS

Newcastle disease virus was isolated from clinically affected grey parrots (*Psittacus erithacus* L.). Despite the slow spread of the disease through the parrots at risk, the isolated virus was found to be fully invasive and virulent for domestic fowls and was typed as mesogenic.

The virus agglutinated fowl, human and guinea pig erythrocytes but not those of cattle, goats, hamsters and horses.

The haemagglutinin was a medium elutor and was heat labile, being inactivated after 10 minutes exposure to 56°C.

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REPRODUCTIVE FAILURE IN RATS DUE TO COPPER DEFICIENCY

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INTRODUCTION

The occurrence of low fertility in cattle due to copper deficiency has been reported by a number of workers. Bennetts, Beck and Harley (1948) reported that the low copper status of dairy cows in an affected region resulted in seasonal anaemia, a high incidence of infertility, decreased production and retarded growth and development of young stock. Allcroft and Parker (1949) also described a disorder affecting dairy cattle on peat land associated with low blood copper levels in adult animals. The condition was characterised by persistent diarrhoea, unthriftiness, low milk production, lowered fertility in cows and a marked degree of stunting in young stock. They observed, however, that pasture analysis showed normal copper contents. Van Koetsveld (1958) reported that copper deficiency can also lead to a low content of milk fat, a fall in milk production, a low fertility and a dry, rough hair discoloured to a greyish tint.

It appears that although low fertility in cattle has been associated with copper deficiency, no systematic work to elucidate the cause of infertility either in cattle or laboratory animals has yet been reported. The present experiment was undertaken with a view to studying the oestrous cycle, conception rate and reproduction in copper deficient rats. It may be mentioned, however, that the original aim was to obtain litters from copper deficient rats with a view to studying the progress of myelination in the central nervous system in the new-born copper deficient rat. Such attempts were thwarted by almost complete reproductive failure, which led to the present experiment.

MATERIALS AND METHODS

In an initial experiment, out of 18 female hooded Lister rats kept on a copper deficient diet, only three rats gave litters. Of these, two delivered dead litters and the third, which gave a litter of nine, started eating its young the moment they were delivered.

Another experiment was then planned to study the oestrous cycle, conception rate and reproduction in deficient rats. Thirty-two female hooded Lister rats 3 to 4 months old were divided into four groups of eight, each having the same approximate aggregate weight but receiving different levels of dietary copper. Group A was kept on a synthetic copper deficient diet of the composition indicated in Table 1. This diet when supplemented with 25 p.p.m. Cu gives good growth with an average liveweight 4 weeks after weaning of 152 g. and a satisfactory reproductive performance; average litter size 9.8, average weaning weight for 1st litters reduced to 8 animals 39.5 g. Groups B, C and D received diets containing 0.5, 1.0 and 2.0 p.p.m. of added copper respectively. The experiment was started on 9th July, 1958 and oestrous cycle studies in all the rats were started on 10th July, 1958 with a view to finding out if the oestrous cycle was regular in the different groups. Vaginal smears, stained by Giemsa, were taken from all the rats in the four groups for one month up to 10th August 1958. During this period, the rats were not mated. From 10th August 1958, normal males were introduced into all the groups. Weekly weighings of all the rats were made. The male rats were fed on a normal stock colony diet.

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TABLE 1

COMPOSITION OF COPPER DEFICIENT BASAL DIET

(Cu content 0·35–0·40 μg/g.)

Purified (low copper) casein	200 g.*
Sucrose	660 g.
Arachis oil	100 g.
Minerals	as below
Vitamins	as below

*Mineral supplement**		*Vitamin supplement* §	
$CaCO_3$	12·4 g.	Thiamine	5 mg.
K_2HPO_4	13·2 g.	Pyridoxine	5 mg.
$CaHPO_4 2H_2O$	3·0 g.	Riboflavin	10 mg.
$MgSO_4 7H_2O$	4·2 g.	p-amino-benzoic acid	10 mg.
NaCl	6·9 g.	Niacin	20 mg.
†KI (A.R.)	0·032 g.	Ca pantothenate	80 mg.
$MnSO_4 4H_2O$	0·21 g.	Folic acid	5 mg.
$ZnCl_2$	0·012 g.	Biotin	0·5 mg.
‡$FeSO_4 7H_2O$	0·130 g.	Vitamin B_{12}	25 μg.
		Calciferol	0·2 mg.
		Vitamin A (alcohol)	4 mg.
		α tocopherol (acetate)	200 mg.
		Inositol	400 mg.
		Choline hydrochloride	1 g.

*Traces of copper removed by solvent extraction procedure (to be published).
†No further purification required.
‡'Specpure', Johnson Matthey & Co., London. (no purification required).
§Roche Products Ltd. (no purification required).

At the end of the experimental period, all the female rats were killed and the internal organs were examined. Particular attention was paid to the reproductive organs in order to detect evidence of abnormalities in the ovaries or uteri. The internal organs and particularly uterine nodules or areas of increased vascular supply were fixed in 10 per cent formol saline, embedded in paraffin wax and sectioned at 3 to 5 μ. Sections were stained by haematoxylin and eosin.

RESULTS

General Observations

It was observed that the oestrous cycle was quite regular in all the groups. Only four rats in group D gave litters; the numbers of offspring varied from 3 to 7. Two of the rats in this group littered on 5th Sept. 1958 and the other two on 6th Sept. 1958.

During the course of the experiment, it was observed that most of the rats in all the groups gained in weight, but after periods of varying length suddenly declined in weight by an average of twenty-five grams. This led to the suspicion that most of the rats had conceived but had failed to deliver, either due to abortion or resorption. It was therefore decided to kill all the rats on 18th Sept. 1958 to look for signs of foetal resorption.

The weight increase of the rats after mating tended to pursue one of three distinct lines (i) a continuous increase in weight after mating suggesting normal foetal growth, (ii) an increase followed by a rapid fall in live weight and (iii) a cyclical increase and fall in live weight suggesting that conception and foetal growth was repeatedly followed by foetal death and resorption. The rats of group D receiving 2 p.p.m. added copper which either produced normal young or were found to be pregnant at slaughter, showed curves of type i. The weight increase of rats of other groups receiving lower copper levels, none of which produced live young, were of patterns ii or iii.

The rats killed after being on their respective diets for 2 months and nine days showed the changes in the genital tract detailed in Table 2. Two of the rats in group B gave unmistakable macroscopic evidence of resorption, since small and large foetuses were found in the same uterus (Fig. 1).

Histopathology

Histopathological examination of the large foetus shown in Fig. 1 revealed vascularisation and leucocytic infiltration of the foetal tissues. The foetal tissues were undergoing active resorption and a large number of macrophages or foreign body giant cells were seen. The foetal tissues were so necrotic that no embryonic organ could be detected. Most of the embryonic tissues after resorption showed a fibrillated albuminous mass (Fig. 2). The smaller uterine swelling obtained from the same rat showed a large fibrinous clot in the uterine lumen.

Histopathological studies of the rats which showed macroscopically only nodules of the uterine wall revealed various stages of resorption and organisation of the necrotic debris. In some of the rats, no sign of foetal tissue was left and its place was taken by dense fibrous tissue after resorption of the embryonic tissues (Fig. 3). The mesometrium showed dilated and congested blood vessels between the muscle walls and the muscle fibres were oedematous. Another rat with uterine nodules seen on microscopic examination showed reparative processes in action after resorption of the foetus. Two grades of fibroblastic cells were seen. In a part of the resorbed embryonic tissue fibroblasts showing collagen production were observed and in another part of the resorbed embryo immature fibroblasts were present. The uterus of another rat on histological examination showed decidual cells which were taking part in organisation after resorption of the embryonic tissues.

TABLE 2

AUTOPSY OBSERVATIONS ON FEMALE RATS KEPT ON COPPER DEFICIENT DIETS

Group	*Number of rats in group*	*Date of start of exp.*	*Date of first mating*	*Level of supplementary copper*	*Date of killing*	*No. of live litters*	*No. of rats at slaughter showing*		
							Pregnancy	*Uterine nodules*	*No macroscopic abnormality*
A ..	8	9 July '58	10 Aug. '58	nil	18 Sept. '58	—	—	5	3
B ..	8	,,	,,	0·5 p.p.m.	,,	—	2	4	2
C ..	8	,,	,,	1·0 p.p.m.	,,	—	—	6	2
D ..	8	,,	,,	2.0 p.p.m.	,,	4	1	3	—

DISCUSSION

It is obvious from the results of these experiments that copper deficiency in rats results in reproductive failure. Out of the 18 rats on a low copper diet in an initial experiment only one produced living young.

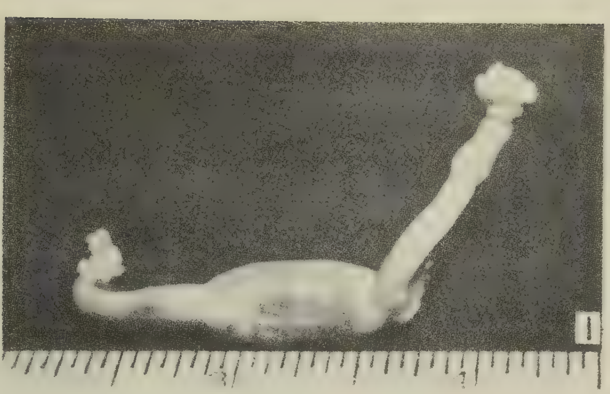
In the second experiment, out of 32 rats on different levels of copper only four of the eight rats in group D receiving 2 p.p.m. supplementary copper gave live litters. Daily vaginal smears over a period of one full month in all the rats showed that the oestrous cycle was regular. The normal oestrous cycle in the copper deficient rats affords reasonable ground for the assumption that such rats would in all probability become pregnant when mated. It may be added that males allowed with the females for mating were frequently changed in order to obviate the possibility of any sterile male being always with the same female rats.

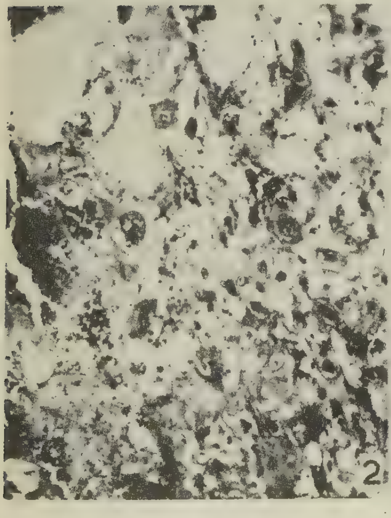
Autopsies of the rats revealed that five rats in group A, four in group B, six in group C and three in group D showed nodules of the uterine walls. These uterine nodules were 2 to 3 mm in diameter, with distinct evidence of increased vascular supply representing areas of implantation of foetuses. The histological examination of these uterine nodules indicated reparative processes in various stages after resorption of the foetal tissues. It should be noted that these rats were destroyed after two months and nine days and during the last one month and nine days they were allowed with the males for mating. It is probable that if the rats were killed earlier when they continued to gain in weight many more rats would have shown embryonic tissues in various stages of resorption. Two rats in group B as mentioned earlier, showed foetuses of different sizes in the uterus which gives an indication that foetal resorption began at different times or that early resorption took place in the case of the small foetus.

Thiersch and Philips (1950) in their experiment on the effect of 4-amino-pteroylglutamic acid (APGA) on early pregnancy reported that rats killed 23 days after mating showed complete resorption of foetuses and placentae. They found only in one rat one previous site of implantation, whereas rats killed on the 14th day of pregnancy and seven days after last APGA injection produced better evidence of foetal damage and death. The results obtained from the present investigation do indicate the desirability of killing the rats from 14 days onward from the date of mating at intervals in order to have a better evidence of foetal damage. It is known that resorption will occur following early embryonic death due to a variety of causes. The reason for the foetal death and resorption in these copper deficient animals has yet to be determined.

CONCLUSIONS

Reproductive failure was first noticed in mature female black hooded rats of 3 to 4 months of age kept on a copper deficient diet





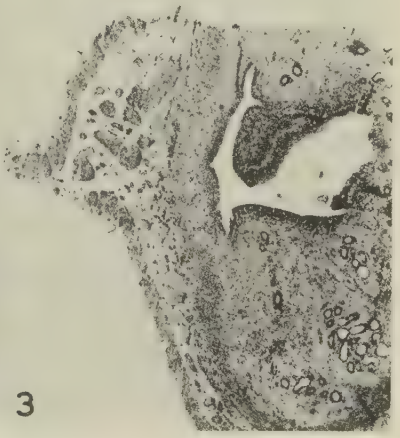


Fig. 1. Uterus of rat from Group B containing one large and one small foetus undergoing resorption.

Fig. 2. Section of large foetus shown in Fig. 1 undergoing active resorption. Note extensive vascularisation and leucocytic infiltration and large number of macrophages. Haematoxylin and eosin (× 80).

Fig. 3. Section through a uterine nodule showing dilated and congested mesometrium. The foetal tissues after resorption have been replaced by dense fibrous tissue. Haematoxylin and eosin (× 27).

in a pilot experiment. Out of 18 rats on a copper deficient diet, only three littered down. The oestrous cycle of all these animals showed no abnormality.

In a second experiment, out of 32 rats on several levels of dietary copper only four of the eight in a group receiving the highest level of 2 p.p.m. added copper produced young. The number of young born varied from 4 to 7. Autopsies carried out on the remaining rats after being fed their respective diets for two months and nine days gave unmistakable evidence of resorption of foetuses. Uterine nodules representing the previous sites of implantation of embryos were found in the majority of the rats.

Microscopic examination of the uteri of rats showing these uterine nodules revealed various stages of reparative processes after resorption of the foetal tissues.

ACKNOWLEDGMENTS

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THE EFFECT OF CHEMOTHERAPY ON THE NUMBERS OF *BACTERIUM COLI* IN THE FAECES OF CALVES

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INTRODUCTION

Although the aetiology of scours in young calves is complex, there is strong evidence that the proliferation of *Bacterium coli* in the alimentary tract plays an important part in some outbreaks. In these outbreaks, the administration of chemotherapeutic agents active against *B. coli* is claimed to have a beneficial effect on the course of the disease. Field observations combined with laboratory studies have supported this (Smith and Crabb, 1956a; Smith, 1958). In fact, the position now is that the primary consideration in recommending any chemotherapeutic agent for use in the treatment of white scours is that it should be active against *B. coli*.

The difficulty in producing the natural disease experimentally has hampered efforts to study and to compare the therapeutic effects of different chemotherapeutic agents in a controlled manner and field trials are fraught with difficulties. Since it is indeed possible that drugs which decrease the *B. coli* faecal count may have a beneficial effect in calf scours and since the faeces of healthy, as well as scouring calves contain large numbers of *B.coli* (Smith and Crabb, 1956b), it was decided to determine the comparative effect of drugs, particularly those commonly used in treating calf scours, on the *B.coli* population of the faeces of healthy calves.

MATERIALS AND METHODS

Calves. Calves remaining healthy throughout the experimental period were used; they weighed 35 to 40 kg. and none was older than three weeks at the conclusion of each observation. Groups of 3 or 4 calves were employed to test a similar number of drugs in turn on each calf within the group. The majority of the experiments were carried out on calves fed cows' milk in a self-contained herd (herd 'A'); the remainder were performed at a second farm (herd 'B') on calves purchased in a market and fed on milk substitute.

Faecal counts of viable B. coli. One gramme amounts of rectal contents were shaken mechanically in 10 ml. of phosphate buffer, pH 7.0, for 3 minutes, filtered through sterile coarse muslin, and viable counts on MacConkey agar performed following the method of Miles and Misra (1938). Incubation of the MacConkey agar plates for 24 hours at 37°C was adequate to permit the enumeration of the colonies of *B.coli* originating from the faeces of the calves, irrespective of the chemotherapy they had been receiving. If very low counts of *B.coli* were anticipated, 1 ml. amounts of the tenfold dilution were spread over a MacConkey agar plate. The *B.coli* counts were performed once daily, two counts being performed before the administration of a drug and an appropriate number afterwards.

Enumeration of drug-resistant B.coli. In some instances trials were complicated by the emergence of *B.coli* resistant to the drug under test. In order to enumerate these resistant organisms, the counts were duplicated on MacConkey agar containing 20 μg./ml. of the particular antibiotic under test.

Estimation of antibiotic contents of faeces. Weighed amounts of faeces were suspended in a small volume of phosphate buffer, pH 7.0, centrifuged at high speed and the supernatant tested for antibiotic content by the punch plate method.

RESULTS

Unless otherwise stated, all the experiments referred to below were uncomplicated by the presence of *B.coli* resistant to the drug under test; reference is made to any resistant strains arising.

Effect of Single Doses of Drugs

Each drug was tested on three to eight calves; the dose given was 10g. of the sulphonamides and 0.5 or 1.0 g. of the other drugs. The greatest and most prolonged reduction in the *B.coli* faecal count was obtained in calves treated with streptomycin or neomycin (5 calves each), such effects being obtained in all calves treated with these drugs. Polymixin treatment consistently resulted in a marked reduction (3 calves). Oxytetracycline,* chlortetracycline,† sulphadimidine‡ and phthalylsulphathiazole, (4 calves each) had a considerable depressing effect on the *B.coli* faecal count although in a small proportion of calves no decrease was found. The administration of 0.5 to 1.0 g. of chloramphenicol to all of six calves had no effect on their *B.coli* faecal count and similar results were obtained in calves given 1.0 g. of either furazolidone (4 calves) or furamazone, (4 calves). The *B.coli* faecal counts of untreated calves, as expected, varied from day to day, but rarely by more than tenfold. A typical result obtained with streptomycin, neomycin, polymixin, oxytetracycline, chloramphenicol and sulphadimidine is illustrated in Fig. 1. The results obtained with chlortetracycline and phthalylsulphathiazole are not shown because they closely resembled those obtained with oxytetracycline and sulphadimidine respectively; neither are the negative results shown which were obtained with furazolidone and furamazone.

Effect of Multiple Drug Dosage

In herd 'B' where purchased calves were kept, many tests were invalidated by the emergence of *B.coli* resistant to the drug under test, but from experiments uncomplicated by resistant *B.coli*, certain conclusions were drawn. As was expected, the daily and twice daily

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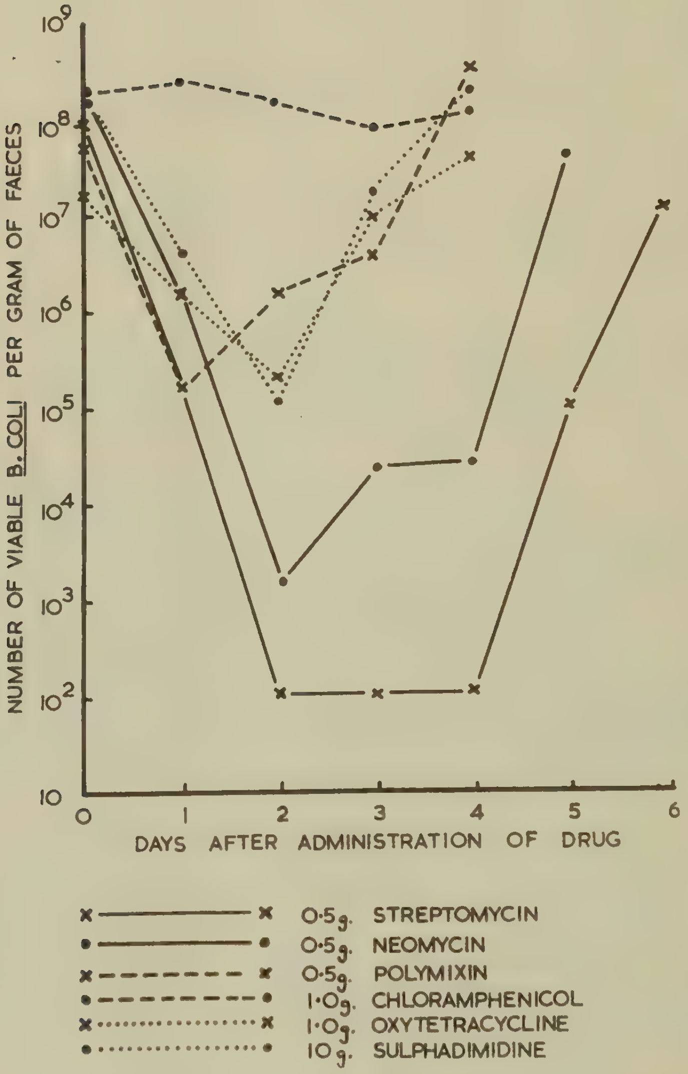
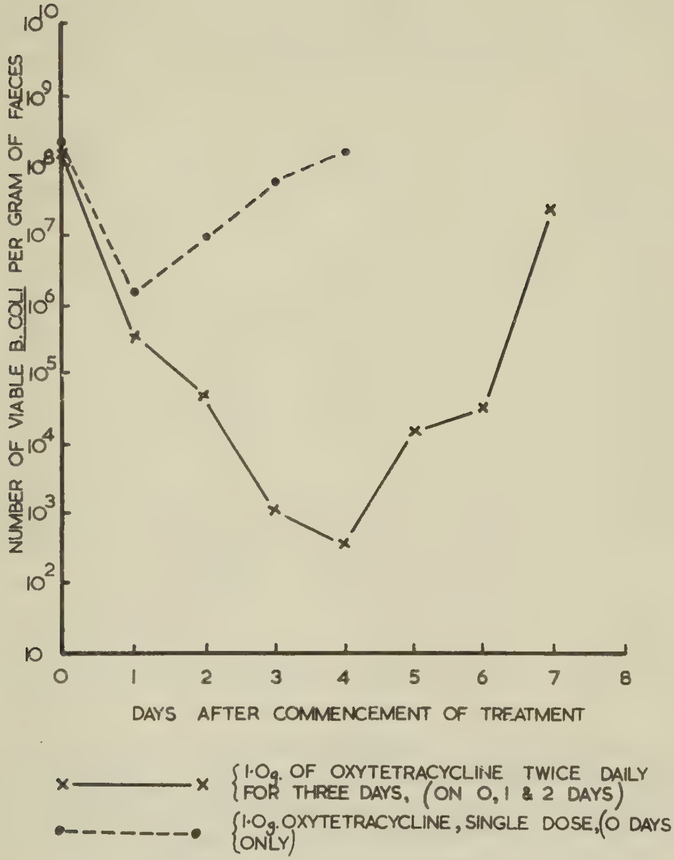
* Terramycin. † Aureomycin. ‡ Sulphamezathine.

Fig. 1



administration of 0.5 to 1.0 g. of streptomycin, neomycin and polymixin had a very marked effect in depressing the faecal *B.coli*, resulting in its absence in some instances. A much greater depressant effect was noted when the two tetracyclines and the two sulphonamides were given twice daily than when they were given as a single dose; a comparison between the effect of giving a single 1.0 g. dose of oxytetracycline and a similar dose, twice daily for 3 days, is illustrated in Fig. 2. No depressant effect was noted when 1.0 g. of chlorampheni-

Fig. 2



col was given once daily; a slight effect was observable when it was given twice daily. Similar results were obtained with furamazone and furazolidone. Multiple dosage of the experimental drugs undergoing development referred to previously had no effect on counts.

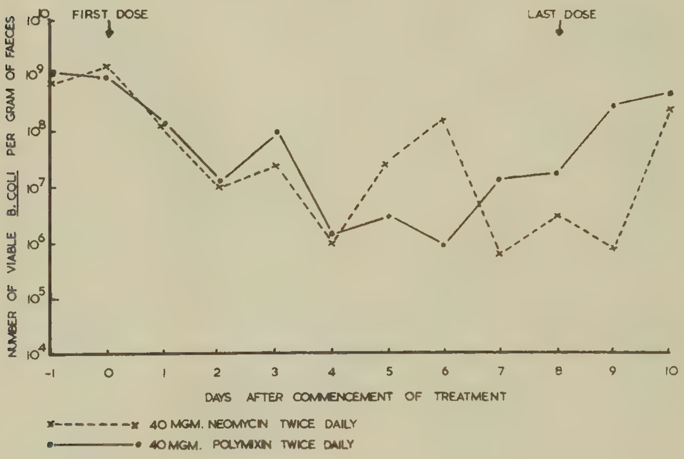
Fig. 3 demonstrates the effect of giving a much reduced dose, 40 mg., of neomycin and polymixin twice daily to calves for 9 days. Although there was a tendency towards depressing the numbers of *B.coli*, the effect obtained did not compare with the marked depression in numbers consistently obtained when one dose of 0.5 g. was employed (Fig. 1).

Presence of Drug in the Faeces of Treated Calves

Because of the much greater depressing effect obtained with the tetracyclines than with chloramphenicol, the antibiotic content of the faeces of calves treated with these drugs was examined. Up to

48 hours after the oral administration of 1.0 g. of chlortetracycline, this drug was found in the faeces in concentrations varying from 10 to 20 μg. per g. of faeces. No drug was demonstrated in the faeces of a similar number of calves given 1.0 g. of chloramphenicol.

Fig. 3



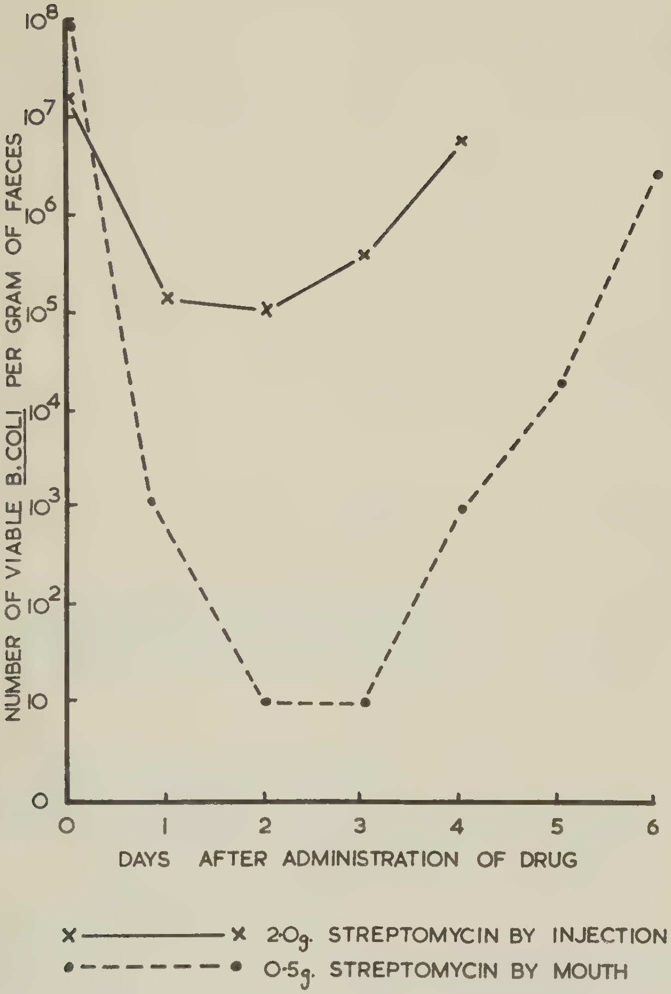
Effect of the Route of Administration of Streptomycin on the B.coli *Faecal Count*

A typical example of comparing the effect of giving 2.0 g. of streptomycin by subcutaneous injection with 0.5 g. of the same drug by mouth is illustrated in Fig. 4. A much greater and more prolonged reduction was obtained in the *B.coli* faecal count of the calf given oral treatment than that given subcutaneous treatment.

Effect of Treatment on the Emergence of Drug-Resistant B.coli.

As mentioned previously, the emergence of drug-resistant *B.coli* during the treatment period invalidated some tests in herd 'B'. This frequently happened in calves treated with streptomycin, oxytetracycline, chlortetracycline, chloramphenicol, sulphadimidine and phthalylsulphathiazole, but never with neomycin, polymixin, furazolidone and furamazone. Some typical examples of the rapidity with which drug-resistant strains become the dominant *B.coli* faecal flora, often as a consequence of only one dose of drug, are shown in Table 1. In some cases the number of resistant *B.coli* present 24 hours after administering the chemotherapeutic agent was greater than the number of sensitive *B.coli* initially present. This resistant population was maintained during the treatment period, but reverted to a partly or mainly sensitive population after a period of time which varied with the extent to which the particular

Fig. 4



drug had been employed in the environment and hence with the proportion of resistant strains present in that environment.

DISCUSSION

It is noteworthy that most of the drugs which are reported to be of value in the treatment of calf scours brought about a great reduction in the numbers of *B.coli* in the faeces of healthy calves to which they were administered. That drugs such as streptomycin, neomycin and polymixin with a bactericidal effect had the greatest depressing

TABLE 1

EFFECT OF CHEMOTHERAPY ON THE EMERGENCE OF DRUG-RESISTANT STRAINS OF *B.COLI*

Drug and dose employed by mouth	*Time after treatment (hours)*	*No. of viable* B.coli $\times 10^3$ *per g. of faeces that grew on media containing*	
		Drug	*No drug*
One dose of 1.0 g. streptomycin	0	1	4,000
	24	1,000	10,000
	48	15,000	15,000
One dose of 0·5 g. streptomycin	0	1	20,000
	24	250,000	250,000
Two doses of 0·5 g. oxytetracycline 12 hours apart	0	0·01	1,000,000
	24	500,000	500,000
Two doses of 1·0 g. oxytetracycline 12 hours apart	0	750	150,000
	24	60,000	60,000
Four doses of 1.0 g. chloramphenicol 12 hours apart	0	0·01	7,500,000
	24	0·05	2,500,000
	48	10,000,000	10,000,000

effect on the *B.coli* population of the alimentary tract was not surprising. The bacteriostatic drugs, such as the tetracyclines and sulphonamides, were much more effective when given twice daily over a period than when given as single doses. The dose level was also important in maintaining a low *B.coli* faecal count. For example, the effect of a course of 40 mg. doses of neomycin and polymixin twice daily on the calf's *B.coli* faecal count was much less marked than when 0.5 g. was employed. A comparison of the effect of giving streptomycin subcutaneously and by mouth, too, left no doubt that the latter route had by far the most marked reducing effect on the *B.coli* faecal counts.

The relatively poor effect obtained with chloramphenicol was, at first, unexpected because this drug is known to be of value in treating white scours. However, the absence of the drug from the faeces of treated calves indicated that it is either completely absorbed or inactivated in the upper intestine so that *B.coli* would be able to multiply uninhibited in the lower gut, the faecal count being little influenced. This resembles the situation in human beings (Seligmann and Wassermann, 1949). Chloramphenicol undoubtedly has an inhibitory effect in the upper gut because, in those calves whose *B.coli* alimentary flora consisted mainly of chloramphenicol-sensitive organisms and a very small proportion of resistant organisms, the administration of the drug rapidly resulted in the resistant organisms becoming the predominant *B.coli* flora. The apparently poor depressant effect obtained with furamazone was unexpected in view of the good results obtained by Henry and Blackburn (1957).

Our clinical experience, however, with this drug, has been disappointing.

Despite the several readily-available drugs that possess considerable activity against *B.coli* in vivo, the chemotherapeutic control of calf scours is seriously handicapped by the emergence of *B.coli* that are resistant to these drugs. The extent of the problem has been referred to elsewhere (Smith and Crabb, 1956a; Smith, 1958). From the point of view of general appplication, a drug must not only be active in vivo, but pathogenic *B.coli* resistant to the drug in question must either be extremely rare or, preferably, not exist or come to exist in the environment of calves. Streptomycin, the tetracyclines, the sulphonamides and chloramphenicol fail in the latter respect. How neomycin and polymixin will fare is problematic. We have experience of neomycin only; to date no neomycin-resistant *B.coli* have been found. Also we have been unable, in the laboratory, to produce *B.coli* cultures resistant to this drug or to polymixin. Whether this will continue to apply when the drug comes into more frequent use is questionable.

It is possible that the techniques employed in the present studies may be of use in screening new drugs and in throwing some light on the manner in which they, and also the more established drugs, may best be employed in the therapy of calf scours. It should be remembered, though, that great differences exist between the state of affairs in the alimentary tract of scouring and of healthy animals and the information they yield must be regarded as being only of a preliminary nature.

CONCLUSIONS

The comparative effect of different drugs, together with the influence of dose level, dose rate and route of administration, on the viable count of *B.coli* in the faeces of healthy calves have been studied. The numerical aspect of the emergence of resistant strains has also been investigated.

The results indicate that such studies may yield useful preliminary information on the value of new drugs and on the best method of employing them, and the more established drugs, in the treatment of calf scours.

ACKNOWLEDGMENTS

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THE EXTRA-NEURAL PHASE OF INFECTION WITH EQUINE ENCEPHALOMYELITIS VIRUS IN THE MOUSE AND GUINEA-PIG IN RELATION TO THE ANTIVIRAL EFFECT OF MEPACRINE

By

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INTRODUCTION

The considerable protective action of mepacrine in the mouse infected with the virus of equine encephalomyelitis (Hurst, Melvin and Peters, 1952) is reproduced in the maturing rat, but not in the guinea-pig, rabbit, chicken or rhesus monkey (Hurst, Snow and Roberts, 1955). Chemical and biochemical studies have not so far afforded an explanation, in terms of differing paths of metabolism or radical differences in distribution of the drug, of this failure of many species to respond to therapy (Goodall, 1956; Snow and Hurst, 1956). We enquired, therefore, whether the course of infection differs materially in the mouse and the guinea-pig. Previous observations on the pathogenesis of equine encephalomyelitis have concerned mainly the manner in which virus invades the central nervous system (Hurst, 1950). Since, however, mepacrine operates chiefly on the extra-neural phases of infection, the present study is confined to the sequence of events before the nervous system is affected and that in animals escaping nervous involvement.

METHODS

Titrations of virus. Mice or guinea-pigs received the virus of Eastern equine encephalomyelitis intramuscularly or intravenously (Hurst, 1950). After bleeding from the heart to prepare pooled defibrinated blood, the vascular system was washed out with saline until the viscera and tissues macroscopically were bloodless; this procedure elimated circulating virus or circulating antibody (see later), as the case might be. Pieces of the organs were pooled either from 3 guinea-pigs or from 6 mice to obtain an overall picture of the amount of virus present at various stages of the disease, or were examined separately to detect individual variations between animals. Serial dilutions of blood or suspensions of the organs at half-logarithmic intervals were injected intracerebrally into groups of 6 mice, and the times at which these died were noted; according to Traub and Schwöbel (1958) this technique is often more sensitive than is infection of tissue-cultures in the estimation of virus. Surviving mice were allotted a number exceeding by unity the last day of specific deaths. The total score for each group of six mice was plotted against the logarithm of the dilution, and a smooth curve fitted by eye; in doing this we were assisted by an "ideal curve" drawn from the results of 6 replicate titrations, within a single day, of the stock virus. The end-point was taken as half way between the maximum and minimum possible scores; it was not, of course, identical with the LD_{50}.

TABLE 1

MEAN TITRES OF EQUINE ENCEPHALOMYELITIS VIRUS IN THE ORGANS OF MICE INFECTED INTRAMUSCULARLY

Organ	*Hours after infection*					
	24	*48*	*72*	*96*	*120*	*144*
Site of inoculation	2·9	2·4	2·7	2·1	2·0	2·2
Blood	2·6	1·3	0·1	N.V.	N.V.	N.V.
Liver	1·7	0·9	0·4	N.V.	N.V.	N.V.
Spleen	1·8	1·5	2·4	1·9	1·3	0·4
Kidney	N.V.	0·7	2·6	1·5	2·6	1·9
Lung	0·4	1·1	—	0·9	—	—
Adrenal	2·2	2·8	1·9	1·1	0·5	—

On each occasion the pooled organs of 6 mice were titrated, and the dilutions of virus giving 50 per cent. end-points in the passage animals were derived as described in the text. The log 10 dilutions are given as positive quantities and represent the means of 6 experiments.

N.V. = No virus detected.

Serum-neutralisation tests. Aliquots of pooled normal mouse serum, or of serum from infected animals were added to equal volumes of successive ten-fold dilutions of virus of known titre. After thorough mixing the mixtures were left for one hour at room-temperature, transferred to iced water and finally injected intramuscularly into groups of 6 mice weighing 17 to 18 g. Each serum was tested thus on three occasions and the results averaged. Similarly an examination was made of 10 per cent suspensions of liver for the presence of antibodies.

Subcellular components. The method of separation is fully described by Snow and Hurst (1956).

RESULTS

Infection in the Mouse

Data for the mouse are more abundant than for the guinea-pig because, in addition to titrations performed specifically for the present purpose, it has been possible to draw upon the control material from a variety of other experiments to be reported separately. Altogether, the wax and wane of virus have been followed in 24 series of mice ranging from 17 to 18 g. at one extreme to full sexual maturity and a weight of 30 to 32 g. at the other. Each series comprised mice of a single sex, more often female. The dose of virus given intramuscularly varied from an approximate LD_{50} to an approximate LD_{95}. As is well known, death in equine encephalomyelitis only ensues when the brain is invaded by the virus (Hurst, 1950); this contingency arises less frequently with advancing age, though the greater resistance of the adult mouse can be partly countered by increased dosage of virus. Thus at 17 to 18 g. a dilution of virus of $10^{-5 \cdot 5}$ may lead to the death of 95 per cent. of the animals, whereas at 30 to 32 g. one of 10^{-2} is needed to ensure a mortality of 50 to 65 per cent. The fact that a variable number of older mice survived in the present experiments does not imply, however, that they escaped systemic infection. Examination of the blood of large numbers of individual mice at 24 and 48 hours after infection revealed abundant virus in all but the rarest instance.

The titres given in Table 1 represent the state of affairs in the young adult mouse infected with a dose of virus sufficient to kill around 65 per cent. Each is the mean of six separate observations, except those for 144 hours which represent only two. The validity of this method of presenting the results was determined by examination of the blood titres at 24 and 48 hours after infection in 16 groups of 6 mice. The titres were significantly correlated with the initial weight (and therefore age) of the mice. When the grouped data were corrected for variations due to this factor and the different times after infection, they showed a normal Gaussian distribution.

At the site of inoculation virus is continuously present for at least 144 hours, by which time the mice not devoted to titration have suffered most of their fatalities. Although titres tend to be higher in animals receiving larger doses of virus, quantitative studies, both in untreated mice and in animals dosed with mepacrine (see later), clearly demonstrate that they are not due solely to virus persisting at the site of inoculation but that growth of virus does occur here. However, histological examination of the site, facilitated by adding a trace of very pure carbon (Darco G60) to the inoculum, disclosed no abnormality not present in controls receiving carbon and saline without virus.

By comparison with many other infections in which virus appears in the bloodstream, the titres reached in the blood by the virus of equine encephalomyelitis are unusually high relative to those in the organs and tissues generally (Hurst, 1936), though experiments many years ago and the more recent ones of Traub (1958) have demonstrated no growth of virus in blood in vitro. In younger animals virus reaches a higher titre and circulates longer than in adults; this fact largely accounts for the higher mortality in the young (Hurst, 1950). In female mice of the age considered in Table 1, the highest titre obtains usually at 24 hours, and little or no virus remains at 72 hours, but occasionally in older mice virus does not reach its maximum titre until the 48th hour; the delayed maximum is not obviously related to the titre at the site of inoculation. In the blood, virus exists only in the plasma. In two experiments, the centrifuged cellular fractions from citrated blood, washed six times in saline, failed to infect mice by the highly sensitive intracerebral route, when the titres of virus in the plasma were $10^{-2 \cdot 1}$ and $10^{-2 \cdot 6}$ respectively. After virus has disappeared from the blood, the serum contains antibodies: in one ad hoc experiment, at 72 hours the serum probably contained traces of antibody, at 96 hours it neutralised 30 to 100 intramuscular infecting doses of virus, and at 120 and 144 hours 300 to 1,000.

With great regularity the virus content of the liver runs very nearly parallel with that in the blood, and on the whole at a distinctly lower level. Because of the very high concentrations of mepacrine attained in the liver, particular attention was paid to the significance of virus in this organ, and detailed studies are described later. By contrast, in the spleen virus increases in amount for a longer period, and the titres are relatively high at a time when virus has disappeared from blood and liver. The titres of pooled organs given in the table conceal the fact that in this respect individual animals vary considerably. Thus at 72 hours virus was present in the spleens of 24 of 32 mice, at 96 hours in 13 of 32, at 120 hours in 15 of 32 (titres $10^{-0 \cdot 4}$ to $10^{-1 \cdot 4}$) and at 144 hours in 0 of 6 mice.

From the kidney virus was always absent at 24 hours. At 48 hours the results were very variable; usually in mice of the age under consideration virus is present in low titre. Later the titre rises, but examination of individual mice at 72, 96, 120 and 144 hours showed the same variability as with the spleen; at 120 hours the kidneys of less than half the mice contained virus ranging in titre from $10^{-0.3}$ to over $10^{-4.0}$. In the lung titres were low. Only 5 of 12 individual mice showed virus at 72 hours, 1 of 12 at 96 hours and 0 of 12 at 120 hours.

In one experiment virus was detected in the ovary at 24 hours and in another in the testis at 72 hours, but not at other times. The figures for the adrenal were obtained in a separate experiment in which 50 mice were examined at each time; the vascular system of these animals was not washed out with saline, but the titres clearly cannot be explained on the assumption that they result from blood retained in the organ. The fatty tissue around the adrenal did not contain virus.

Voluntary muscle taken from the side opposite to the site of inoculation yielded extremely variable results. Sometimes apparent titres as high as $10^{-2.4}$ were obtained; at other times no virus was detected at any stage. Histological examination of similar pieces of muscle demonstrated the inconstant presence of small lymph nodes; it is not known whether this is the explanation of the variable results. Virus was demonstrated in the peripheral lymph nodes—not necessarily those most nearly adjacent to the site of inoculation—but the tiny amounts of tissue available did not permit a quantitative determination. At 72 and 96 hours the cardiac muscle yielded virus in rather less than a quarter of the animals.

If virus be administered intravenously, thereby eliminating a localised site of inoculation, the course of events is very similar. Depending on the dose of virus, within three hours the blood is either non-infective or the titre of virus is reduced to approximately one-thousandth of that obtaining within five or ten minutes of injection. By six hours virus has re-appeared in the blood and then rises to a maximum titre at or before 24 hours. The titre of virus in the liver follows that in the blood. The titres in spleen, kidney, lung and muscle are higher at 24 hours than after intramuscular injection of virus; thereafter they follow the trend of those in Table 1, with the same animal-to-animal variation in the later stages.

Significance of Virus in the Liver

It is evident that viral titres in the liver differ from those in other organs in being much more closely dependent on the level of virus in the blood, suggesting perhaps that virus does not multiply in this organ or at least only slightly and transiently. Before this fact was fully apparent an attempt had been made to localise the virus in the liver cell, because it is here that mepacrine reaches its highest concentration and hence might be expected to exert its most powerful antiviral effect. Accordingly subcellular fractions were prepared by the method previously described (Snow and Hurst, 1956) and the virus in each was estimated. The results were surprising in that well over 90 per cent. of the virus appeared to be in the nuclear fraction, with little or none in the mitochondria or microsomes, and only a modest amount in the supernatant remaining after all the subcellular components had been removed. By adding virus to isolated nuclei it was shown that adsorption could not account for its apparent nuclear localisation. Yet a similar experiment with infected brain did not show virus predominantly in the nuclear fraction. Meanwhile, evidence from the titrations was beginning to suggest that virus in the liver might merely represent particles removed from the blood by phagocytosis.

The purity of the various subcellular components of the liver cells had previously been checked in smears stained with Giemsa, and the nuclear fraction had appeared satisfactorily "clean". The method of preparation was now repeated using the liver of an animal which had been dosed intravenously with staphylococci. On staining smears of the nuclear fraction by Gram's method, it was at once evident that the material was contaminated with a few intact Kupffer cells and with many large pieces of Kupffer-cell protoplasm recognisable by the presence of massed cocci, a defect in the technique of separation which has escaped observation by previous workers. It seemed, therefore, that if in equine encephalomyelitis the virus in the liver exists mainly in the Kupffer cells which remove it from the blood, all the facts would be explained. An attempt was made to confirm this supposition by demonstrating virus in the Kupffer cells with fluorescent antisera. A high-titre antiserum to the virus of equine encephalomyelitis was coupled to Lissamine Rhodamine RB 200 by the method of Chadwick, McEntegart and Nairn (1958), and an anti-globulin rendered fluorescent by fluorescein was also applied to sections previously treated with antiviral serum. No virus was convincingly demonstrated by either method in sections of infected liver, though the same sera have shown virus in infected mouse brain and in infected chorio-allantois. In these other tissues, of course, virus is present in much higher titre than in the liver, and the only conclusion is that insufficient is present in the latter to permit demonstration by fluorescence techniques.

The early disappearance of virus from the liver probably is not linked with the advent of circulating antibodies; not only does hepatic virus vanish at too early a stage and at a time when titres in the spleen and kidney are still rising, but, as has been explained, the blood had previously been removed by perfusion. The experiments also failed to detect neutralising antibodies in 20 per cent. suspensions of the unperfused livers of mice sacrificed at 96 and 120 hours.

On the whole, present evidence does not suggest that virus multiplies freely in the liver, but that virus detected here represents that taken into the Kupffer cells by phagocytosis during the period of viraemia. Thereafter, it rapidly disappears, possibly by reason of the active metabolism of foreign protein believed to occur in this situation (Biozzi, Halpern, Stiffel and Mouton, 1958).

Effect of Mepacrine in the Mouse

Titrations similar to those in Table 1 carried out in mice which had received a single oral dose of 400 mg./kg. mepacrine 24 hours before infection showed almost complete suppression of viral increase. Except at the actual site of inoculation, where low and delayed titres were obtained ($<10^{-1}$), the only virus detected was in the spleen, where the amount was too low for accurate estimation, and in the adrenal where titres of $10^{-0\cdot4}$ and $10^{-0\cdot7}$ existed at 48 and 72 hours respectively. In animals dosed similarly and infected by the intravenous route, the drug did not affect the rate of removal of virus from the bloodstream, but once it had gone the virus did not return as it did in the undosed mice.

In most large groups of mice treated with the above dose of mepacrine, a small number of animals fail to be protected and succumb to encephalitis, usually after a protracted incubation period. [The great majority of these failures may be eliminated by a second dose of mepacrine 48 hours after the first]. In these mice virus reaches a higher titre at the site of inoculation after an initial delay of two or more days, incidentally thereby demonstrating that local multiplication does occur; viraemia begins on the third or fourth day and the titres of virus in the spleen and kidney ultimately reach much the same level as in untreated mice. Of course, should such an animal be included by chance in a group used for titration the result of the latter is vitiated and must be discarded.

With suboptimal doses of mepacrine (100 to 200 mg./kg.) the appearance of virus at the site of inoculation is frequently delayed until 48 hours, after which it may or may not rise to levels near those in the controls. The average titres of virus in the blood and viscera are nearly always lower than in the controls, and the proportion of individual mice showing no virus in the spleen, kidney, etc. at 72 hours and later is much higher; virus is not isolated from the muscles generally. A close parallel exists between the maximum blood titre and duration of viraemia on the one hand and the mortality from encephalitis in parallel groups on the other. A relation between mortality and the amount of virus at the site of inoculation is far less evident.

In vitro experiments suggested that in no area is a direct inactivation of virus by mepacrine likely. Thus in one test amounts of virus ranging from 10 to 1000 LD_{80}, as measured by intramuscular injection of 19 to 20 g. mice, were not diminished in activity by a (final) concentration of mepacrine of 0·5 mg./ml. In this experiment one set of controls received virus plus water in one leg and an equivalent amount of mepacrine into the other leg: the other water and virus controls and the test mixture animals received an injection of water into the opposite leg.

Infection in the Guinea-pig and the Lack of Effect of Mepacrine

Table 2 presents similar data for the guinea-pig with, in

TABLE 2

MEAN TITRES OF EQUINE ENCEPHALOMYELITIS VIRUS IN THE ORGANS OF GUINEA-PIGS INFECTED INTRAMUSCULARLY

Organ	*Hours after infection*				
	24	*48*	*72*	*96*	*120*
Site of inoculation	1·5 (0·8)	2·1 (1·5)	2·4 (Tr.)	—	N.V. – 1·0
Blood	1·8 (2·5)	0·7 (1·3)	0·1 (0·7)	N.V. (Tr.)	N.V.
Liver	1·2 (0·7)	0·6 (0·7)	0·1 (0·4)	N.V.	N.V.
Spleen	0·8 (0·6)	0·2 (0·3)	0·2 (0·2)	N.V. (0·2)	N.V. – 1·0
Kidney	N.V. (N.V.)	N.V. (0.2)	0·1	Tr.	T.V. – Tr.
Lung	0·3 (N.V.)	N.V. (0·2)	N.V.	N.V.	N.V.
Adrenal	1·6 (2·5)	1·7 (1·9)	1·1 (2·5)	1·6 (2·8)	N.V. – 3·0
Muscle	N.V.	N.V.	N.V.	N.V.	N.V.
Lymph nodes	1·8	—	1·0	—	N.V. – 1·9

On each occasion but one the pooled organs of 3 guinea-pigs were titrated. The figures are means except those at 120 hours which denote the range of values in individual guinea-pigs. The figures in parentheses are those for duplicate guinea-pigs given orally 300 mg./kg. mepacrine 24 hours before infection and 150 mg./kg. 4 hours before. (The guinea-pig will not tolerate 400 mg./kg. in a single dose, as will the mouse).

N.V. = No virus detected. Tr. = Amount of virus too small for accurate estimation.

parentheses, the titres in duplicate animals dosed with mepacrine. The guinea-pigs weighed around 250 g. and physiologically were younger animals than the mice in Table 1; the increase in virus should therefore have been greater (Hurst, 1950). On the whole, however, though following much the same trend as in the mouse, the titres were lower in the blood and viscera, so that in the guinea-pig mepacrine does not have to contend with a more devastating infection. While treatment appeared somewhat to restrict increase of virus at the site of inoculation, the effect on virus circulating in the blood was the opposite. Otherwise, it had little positive effect except in the adrenals, where viral titres were consistently higher in treated animals. As matters of interest it will be noted that virus was never detected in voluntary muscle away from the site of inoculation, and that in the last stages of the disease animal-to-animal variation in the viral content of the viscera occurred as in the mouse. Finally virus was not demonstrated in the ovary, nor in the pituitary unless the animal had developed encephalitis.

While the total data are what might be expected from the failure of mepacrine to have any beneficial effect on equine encephalomyelitis in the guinea-pig, they reveal no peculiarity in the infection in this species which would explain its insusceptibility to the action of the drug.

DISCUSSION

This communication describes the detailed evolution of the systemic phase of equine encephalomyelitis in the mouse and in the guinea-pig. Infections in the two species are fairly comparable except that titres generally seem to be rather lower in the latter. The data do not provide any explanation as to why mepacrine possesses therapeutic properties in the mouse but not in the guinea-pig, and the information obtained relates largely to the untreated disease.

Since the work was completed, Traub (1958) has reported a rather similar investigation in mice. While agreeing in broad outline with the foregoing, his data differ in certain details; how far these differences are due to the use of a different strain of virus and how far to other factors is uncertain. Although Traub's animals were not perfused to remove virus present in the blood, it is obvious that the differences are not attributable to this omission. The method of subcutaneous infection adopted by Traub does not lend itself readily to study of the fate of virus at the site of inoculation; in his animals early and considerable growth of virus occurred in the bone marrow, which was not examined in our experiments. The ovaries also appear to have contained virus more constantly and in larger amount than in our mice, though considerable differences were obtained between two series of animals examined at different times of the year and Traub suggests that seasonal factors may operate. Traub's results demonstrate the transient appearance ("growth") of virus in the liver, as well as the persistence of virus in the spleens and kidneys, together with the considerable variations in the amount of virus present at any time in animals treated identically.

CONCLUSIONS

The course of the systemic phase of infection with equine encephalomyelitis in the mouse and the guinea-pig is traced in detail. No peculiarity is revealed in the latter host to explain its insusceptibility to the action of mepacrine.

During an attempt to localise virus in the subcellular components of the liver cell, a hitherto unsuspected technical flaw was discovered in the method of separation; the nuclear fraction was contaminated heavily with large fragments of protoplasm of ruptured Kupffer cells. The presence of virus in the liver can best be explained on the assumption that it results from phagocytosis of virus by the Kupffer cells during the stage of viraemia, and does not represent an unequivocal growth of virus in this organ.

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ERRATUM

E. F. Annison, K. J. Hill, D. B. Lindsay, and R. A. Peters,
Vol. 70, No. 1, Page 145.

Legends should read:

Fig. 1. Blood acetate △

Fig. 2. Injection of sodium fluoroacetate ▲
Blood glucose ▼

FLUOROACETATE POISONING IN SHEEP

By

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INTRODUCTION

Poisoning of livestock by the ingestion of the South African plant *Dichapetalum cymosum* has long been recognised. The toxic principle of this plant is fluoroacetate (Marais, 1944). More recently the introduction of sodium fluoroacetate as a rat and rabbit poison has provided another source of this toxic substance which may be potentially dangerous to the grazing animal. These facts justify a close study of fluoroacetate poisoning in ruminants since, despite a considerable literature on the biochemical nature of fluoroacetate poisoning in laboratory animals (Peters, 1952), there is little reliable information on its action and toxicity in the larger domestic animals. The most urgent problem however is to obtain an antidote. In some animals, acetate is protective in fluoroacetate poisoning. In ruminants, however, acetate is a major product of fermentation in the rumen, and the concentration of acetate in the blood of these animals is usually 3 to 10 times that of non-ruminants. It was not, therefore, anticipated that acetate would prove to be an effective antidote in sheep, but some protective action of acetate has been demonstrated in preliminary experiments which are described in this paper. Data on the toxic dose of fluoroacetate in sheep and some of its biochemical effects are also reported.

MATERIALS AND METHODS

Animals. Clun Forest and Welsh Mountain wethers and ewes kept under animal house conditions were used in this investigation. They were fed each morning with 700 g. of chopped hay and consumed the ration within four hours. On the day of experiment food was withheld. During long term experiments the animals were fed as usual. Several of the animals were fitted with rumen fistulae for the addition of fluoroacetate directly to the rumen; in other animals this was done by means of a stomach tube. Blood samples were removed from the jugular vein and clotting was prevented by heparin. Injections of fluoroacetate were given into the jugular vein.

Sodium fluoroacetate. This was obtained from Monsanto Chemicals Ltd. and used as a solution in 5 ml. distilled water. It contained no detectable inorganic fluoride and was 100.1 per cent sodium fluoroacetate on the basis of its fluorine content. Dose rates were calculated in terms of the free acid.

Monoacetin. A commercial preparation (B.D.H.) was used.

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Methods of Estimation

Blood sugar. Blood sugar was estimated initially by the copper-reduction method of Somogyi (1952) coupled with the colorimetric procedure of Nelson (1944). In later experiments the more specific glucose oxidase method of Huggett and Nixon (1957) was used. Blood glucose values were about 5 to 10 mg/100 ml less when measured by the latter method.

Blood lactate. The method of Barker and Summerson (1941).

Blood ketones. The method of Bakker and White (1956).

Plasma non-esterified fatty acids (NEFA). The method of Dole (1956) was used, except that extraction was made in the presence of phosphate buffer pH 6.0 instead of 0.1 N sulphuric acid. Recovery experiments have shown that acetate and lactic acid are not estimated in this fraction when this modified technique is used.

Blood volatile fatty acids (VFA). The method of Annison (1954) was used.

Tissue citrate. The method of Taylor (1953).

Liver and muscle glycogen. The method of Good, Kramer and Somogyi (1933).

TABLE 1

EFFECT ON SHEEP OF SINGLE OR DOUBLE DOSES OF FLUOROACETATE

The second dose proved lethal in all cases

Sheep	*Dose rate (mg./kg. body wt.)*		*Route of administration*	*Interval 1st to 2nd dose*
	1st dose	*2nd dose*		
G. 32	0·2	0·2	Intravenous	2 days
43	0·2	0·2	,,	11 days
35	0·3	0·4	Rumen fistulae	4 days
78	0·2	0·3	Stomach tube	1 month
108	0·2	0·3	Stomach tube	
112	0·2	0·3	Stomach tube	
	All 6 lived	All 6 died		
H.85	0·4	Fatal	Intravenous	
D. 53	0·4	Fatal	Intravenous	

RESULTS

In the first experiments, dosage of sheep with 0.4 mg. fluoroacetate/kg. body weight proved fatal within 4 to 6 hours, but, irrespective of the route of administration, doses of 0.2 and 0.3 mg/kg body weight produced only slight hypersensitivity. Subsequent experiments, however, indicated that after recovery from a sub-lethal dose, the animals exhibited an increased susceptibility to fluoroacetate, as indicated in Table 1. Thus two sheep (G32 and G43) which received 0.2 mg/kg. intravenously showed only slight symptoms, and yet an identical dose given after 2 and 11 day intervals respectively proved rapidly fatal. Similarly 0.3 mg./kg. which was also a sub-lethal dose was found to be fatal if administered to animals which had previously received a sub-lethal dose.

This behaviour of the sheep is the opposite of that of the rat where a dose which itself is not fatal provides some protective effect towards further doses for some 24 to 36 hours (Chenoweth, 1949).

TABLE 2

EFFECTS OF SUCCESSIVE SUB-LETHAL DOSES OF SODIUM FLUOROACETATE ON SHEEP

Exp.	*Sheep No.*	*Dose given*	*Route*	*Results*
1.	Clun	0·05 mg./kg. daily 14 days	Via rumen fistula	No ill effects.
2.	B.91 (1)	0·1 mg./kg. at intervals 3, 4, 4, 3, 3, and 4 days.	I.V.	Circulatory collapse, very ill after 5th dose. Recovered, no symptoms with succeeding 2 doses.
3.	B.91 (2)	0·25 mg./kg. at 3 day intervals.	I.V.	One month after experiment above. Very ill after first dose, hypersensitive tremors. Died 1 hr. after 2nd dose.
4.	B.14	0·1 mg./kg. at 3 day intervals.	I.V.	Died suddenly 9 hr. after 4th injection.
5.	D.48 (i)	0·1 mg./kg. at intervals 3, 4, 4, 2 days.	I.V.	12 hr. after last of these doses animal tremulous, may have had minor convulsive attack. Fully recovered a further 12 hr. later.
6.	D.48 (ii)	0·1 mg./kg. at intervals 2, 3, 3, 3, 3 days	I.V.	One month after experiment above animal died suddenly after 5th injection. No convulsion.

This cumulative effect was observed in additional experiments in which smaller doses of fluoroacetate were given at regular intervals (Table 2). Sheep B.14, for example, was given 0.1 mg./kg. at 3 day intervals. No symptoms were observed for the first three doses, yet a fourth dose of the same amount proved fatal. Of course, with these small divided doses, it is clear that if the total dose administered is considered, then the animals may tolerate more than a dose which would be fatal if administered as a single dose. Sheep B.91 for example received fluoroacetate equivalent to 1.2 mg./kg. before dying, and sheep D.48 1.1 mg./kg.

The intra-ruminal administration of 0.05 mg./kg. daily for 14 days in one sheep was without effect. This one experiment was not in agreement with the results of Jarrett and Packham (1956), but differences in breed of sheep and diet probably accounted for this discrepancy.

Striking changes were observed in the blood lactate and glucose concentrations following the administration of a lethal dose of fluoroacetate (Fig. 1). Within 90 min. of injection the blood glucose had risen from 40 to 140 mg./100 ml. and the blood lactate from 10 to 70 mg./100 ml. During this period, the blood VFA

Fig. 1

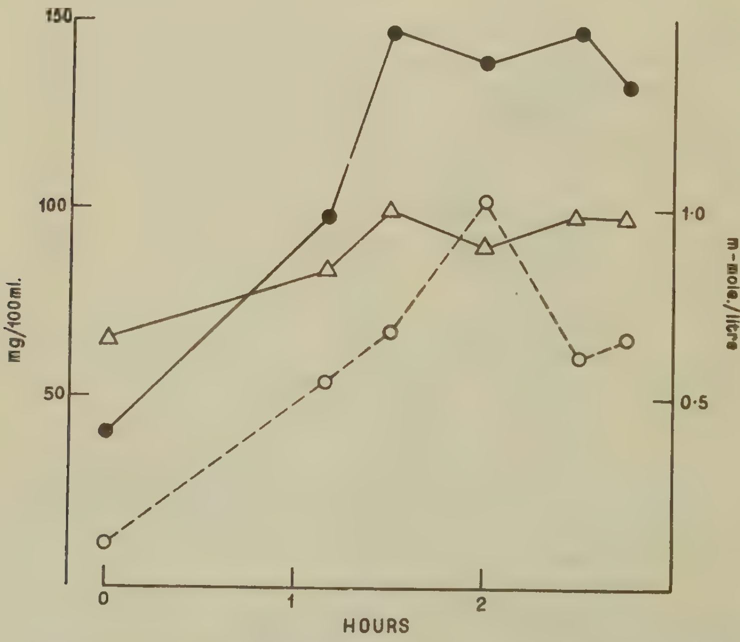

Fig. 1. Changes in some blood constituents after giving a single lethal intravenous injection (0·4 mg/kilo) of sodium fluoroacetate (at time 0). Blood acetate (mM/1) ▼; Blood glucose (mg/100 ml) ●; Blood lactate (mg/100 ml) O.

concentration increased from 0.7 to 1.0 m.mol/l although VFA production in the rumen was presumably minimal since the animal had been deprived of food for about 24 hours.

Although the increase in blood glucose and lactate concentrations was always found in experiments where a single injection of fluoroacetate proved fatal, the time sequence of these events showed some variation in different experiments. Table 3 for example records an experiment in which the increased levels of blood glucose and lactate only occurred 30 to 40 mins. before death. A notable feature of this experiment was the striking rise in blood VFA concentration and a parallel rise in the plasma NEFA. There was also a transient slight increase in the blood ketone body concentration in this particular experiment although in other experiments this change did not occur.

TABLE 3

BLOOD CHANGES AFTER A LETHAL DOSE OF FLUOROACETATE

Time after injection (hr.)	*Blood glucose mg./100 ml.*	*Blood lactate mg./100 ml.*	*Ketone bodies as acetone mg./100 ml.*	*NEFA m-mole/litre*	*VFA m-mole/litre*
0	30	12·9	2·0	0·67	0·46
1·5	40	12·2	2·0	0·61	0·67
2·5	37	9·3	3·7	1·24	0·91
4·0	63	14·5	3·6	1·79	1·35
5·0	44	11·2	4·5	1·87	1·51
5·75	66	39·4	3·0	1·51	1·23
6·5	130	126	2·5	0·99	2·20

Successive sub-lethal doses of fluoroacetate sometimes produced somewhat similar results in that each injection produced a sharp rise in the level of blood glucose and lactate (Fig. 2). This rise was however not persistent and both glucose and lactate had usually returned to their pre-injection levels within 12 hours of injection. No significant changes were observed in the concentration of blood VFA, NEFA and ketone bodies.

In other experiments in which animals received successive sub-lethal doses of fluoroacetate, marked increases in the concentration of blood glucose and lactate following each injection did not occur (Table 4). In one case a blood sample obtained only 2 mins. before death showed blood glucose and lactate concentrations within normal limits (58 and 16 mg. per cent. respectively). This raises the question whether any such changes are due to secondary factors like sympathetic stimulation.

Fig. 2

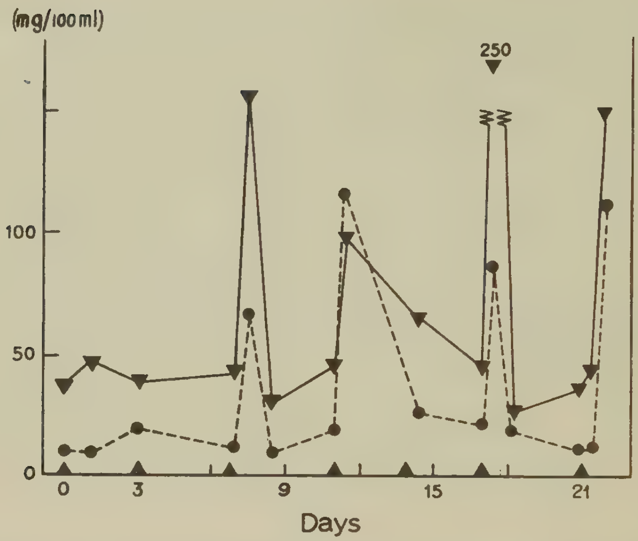

Fig. 2. Changes in blood glucose and blood lactate following successive administration of a non-lethal dose of sodium fluoroacetate (0·1 mg/kilo). Injection of sodium fluoroacetate △; Blood glucose ●; Blood lactate ○.

TABLE 4

CHANGES IN BLOOD CONSTITUENTS

Repeated injections of 0·1 mg./kg. body wt. sodium fluoroacetate at intervals of three days

Fluoroacetate injection	*Sampling time (hr. after injection)*	*Blood glucose*	*Blood lactate*	*VFA*	*NEFA*
1st ..	0	28	16	1·7	0·22
	5	39	15	1·4	0·20
2nd ..	0	47	23	0·8	0·18
	6	52	18	0·7	0·20
3rd ..	0	47	26	0·7	0·85
	5	20	12	0·5	0·24
4th ..	0	36	3	0·4	0·60
	5 Died at 8	65	11	0·7	0·58

Examination of various tissues removed immediately after death showed that citrate accumulation occurred (Table 5) as has been demonstrated in the rat, with the exception that amounts in the heart were relatively much increased; lower increases were found in kidney, spleen and diaphragm. Enhanced concentrations of citrate in the tissues were also found in the one animal from which it proved possible to obtain samples of tissue immediately after death after receiving repeated injections of sub-lethal doses of fluoroacetate.

TABLE 5

CITRATE CONCENTRATIONS IN THE TISSUE OF SHEEP

Sheep	*Heart*	*Kidney*	*Liver*	*Brain*	*Spleen*	*Diaphragm*
Non-poisoned ..	0·21	0·26	0·11	0·24	0·18	0·33
Given 0·4 mg. ..	2·74	0·66	0·11	0·28	0·77	1·13
Fac/kg.	1·61	0·73	0·04	0·23	0·53	0·98
Given 0·1 mg./kg. at 3 day intervals	2·46	1·06				

Values are in μ moles/g. wet weight of tissue. Samples of tissue were taken within 15 minutes of death. The non-poisoned animal was shot, but death occurred spontaneously in the poisoned animals.

Analysis of the liver and muscle of animals which had received a single lethal dose of fluoroacetate and which exhibited a marked hyperglycaemia before death showed marked glycogen depletion. This was not so in the experiments in which repeated sub-lethal doses of fluoroacetate were given and in which there was little change in the blood glucose and lactate concentrations (Table 6).

With the above data in mind and since it was known that acetate affords some protection against poisoning by fluoroacetate in some animals (Chenoweth, Kandel, Johnson and Bennett, 1951) the protective effects of artificially raised intra-ruminal levels of acetate were investigated. The results of three experiments are summarized

TABLE 6

CHANGES IN GLYCOGEN LEVEL IN LIVER AND MUSCLE OF FLUOROACETATE POISONED SHEEP

	Liver glycogen	*Muscle glycogen (abdominal)*
Animal given 0·1 mg./kg. fluoroacetate at 3 day intervals. No blood sugar rise before death.	3·5	0·45
Animal given single dose of 0·4 mg./kg. fluoroacetate. Hyperglycemia and hyperlactacidaemia before death.	0·07	0·11

Glycogen expressed as g. glucose/100 g. wet tissue.

in Table 7. Continuous intra-ruminal administration for 24 hours of acetate sufficient to maintain the concentration of acetate in rumen fluid at 140 m.mol/l protected one animal against a dose of 0.4 mg./kg. of fluoroacetate. Blood acetate levels of 2 to 3 m.mol./l were found throughout this period. In another animal in which larger amounts of fluoroacetate were given, the administration of large doses of acetate before and for long periods after dosage with fluoroacetate caused a marked delay in the onset of the symptoms of toxicity but did not prevent the ultimate death of the animal. Acetate administration for 6 hours after injection of fluoroacetate followed by monoacetin injected intramuscularly (20 m.mol/kg. body weight) in one animal was not demonstrably more effective in conferring protection against fluoroacetate (Table 7). In view of these unpromising results this approach was not continued.

TABLE 7

PROTECTIVE EFFECT OF ACETATE IN FLUOROACETATE POISONING

Exp.	*Sheep*	*Dose of sodium fluoroacetate mg./kg.*	*Acetate admin.*	*Results*
1	A	0·4	500 mM. with the poison 100 mM. per 30 min. for 7 hrs.	Hyperexcitability after 24 hrs. No other signs.
2	A	0·8	200 mM. every 90 min. for 6 hrs.	Collapsed after 24 hrs. Dead 2 hrs. later.
3	B	0·8	As above. After last dose 22 mM monoacetin subcutaneously	Found dead 14 hrs. later. Probably dead at least 4 hrs. before this.

DISCUSSION

Previous estimations of the lethal dose of fluoroacetate for sheep have indicated that it lies within the range of 0.25 to 0.5 mg./kg. when given by mouth (Jensen, Tebiska and Ward, 1948; Meldrum, Bignell and Rowley, 1957). The present finding that the fatal dose was 0.4 mg./kg., calculated as the free acid, is essentially in agreement with these results.

From the relatively small number of results obtained it appeared that when animals received successive sub-lethal doses of fluoroacetate the outcome depended on the size of the dose and the time intervals between administration. If 50 to 75 per cent. of a lethal dose was given the animals became more susceptible to a further dose and 50 per cent. of the lethal dose then proved fatal. This cumulative effect was not at first appreciated and in our preliminary investigations on the toxic dose of fluoroacetate, animals which had received small amounts of fluoroacetate without showing any obvious effects were used a second time for toxicity tests. Even though several weeks elapsed between the administration of the two doses the animals showed increased sensitivity to fluoroacetate and 0.2 mg./kg. body weight proved rapidly fatal.

The cumulative effect of fluoroacetate has been noted in other species and has been attributed largely to the slowness with which circulating fluoroacetate is cleared by the kidney (Chenoweth, 1949). This might also explain why a high concentration of acetate in the blood is only partially effective in preventing poisoning. If one assumes that acetate protects by preventing the formation of fluoroacetyl-CoA then this can only occur as long as the level of circulating acetate is high. If the concentration of circulating acetate falls, even after remaining high for a long period, there is still sufficient circulating fluoroacetate present to give rise to fluoroacetyl-CoA and fluorocitrate and hence cause death of the animal. Supporting this notion is the evidence of Jensen *et al.* (1948) that the time elapsing between administration of fluoroacetate and death is roughly inversely proportional to the size of dose given.

A further point which may be relevant to any explanation of the cumulative effects of fluoroacetate relates to the increased levels of tissue citrate which are a concomitant of fluoroacetate poisoning. This increased concentration has been noted even when sub-lethal doses of fluoroacetate are administered and no obvious symptoms are seen. Thus Lindenbaum, White and Schubert (1951) showed that there was a general enhancement of citrate levels in the tissues of rats which had received daily doses of fluoroacetate, although there were no toxic effects. Their data further indicated that there was a certain critical level of citrate accumulation. Once this was reached in a vital organ, e.g. the heart, fatal symptoms developed. We did not demonstrate such rises in citrate following sub-lethal doses in the sheep, but it seems quite likely that they did occur. Certainly such changes would fit in satisfactorily with the observations showing that when 0.1 mg./kg. fluoroacetate was given every three days, periods up to three weeks were required before death occurred. It seems most unlikely that slow clearance of fluoroacetate by the kidney could explain such observations.

It is of interest that Jarrett and Packham (1956) found that 0.05 mg./kg. daily of fluoroacetate given intra-ruminally caused death within two to three weeks in sheep which were fed on wheaten chaff. No ill-effects occurred if the animals were fed on lucerne chaff or if the wheaten chaff was supplemented with gluten. This might be related to the protective effects of acetate since undoubtedly there would be much greater production of VFA from diets of lucerne and wheaten chaff plus gluten.

The marked hyperglycaemia observed in many of our experiments indicated that fluoroacetate diabetes which has been described as occurring in the rat might also occur in the sheep. The concomitant increase in blood lactate was also in accord with the theory that there was inhibition of glucose oxidation in the tricarboxylic acid cycle. However, the precipitous decline observed in the glycogen content of the liver and abdominal muscle might equally well have been the result of rapid mobilisation of tissue glycogen and this itself may have accounted for the hyperglycaemia, without there necessarily being any inhibition of glucose utilisation. Extensive glycogenolysis has also been reported to occur in the rabbit (Chenoweth, 1949). The effect is reminiscent of adrenalin release or intense sympathetic stimulation and in this connection it is of interest to note that Engel, Fredericks and Cole (1956) have emphasised the shock-like condition of fluoroacetate poisoned rats.

The increase in the short and long chain fatty acid content of the plasma seems likely to reflect interference with the utilisation of these substances by fluorocitrate. Since however, Gordon and Cherkes (1956) has shown that in man plasma NEFA increases after injection of adrenalin, and Lindsay (unpublished) has shown that this is true also of the sheep, it is conceivable that the rise in fluoroacetate poisoning may be merely a consequence of adrenalin release or sympathetic stimulation as for glucose. However, the difference in time-course of changes in plasma NEFA and blood sugar made it unlikely that they arise from the same cause (i.e. sympathetic stimulation). Since the rise in NEFA and VFA correspond well in time and since the latter almost certainly arises from an inhibition of utilisation, it would seem plausible to suppose that the same is true of the long chain fatty acids.

It is of interest to calculate the possible inhibition of acetate oxidation. Lifson and Swanson (1953) found in the mouse that fluoroacetate inhibited about 50 per cent. of acetate oxidation, a figure confirmed by Gal, Peters and Wakelin (1956) for the rat. Using the data for the rise in blood VFA, mainly acetate, following fluoroacetate poisoning, the values of Annison and Lindsay (1958) for acetate utilisation, and the fraction of acetate utilised which is oxidised in the normal sheep, it may be calculated that fluoroacetate did not stop more than 25 per cent of normal acetate oxidation. The relatively lower figure in the sheep may reflect a partial protection by the circulating acetate normally present.

In contrast to the blood NEFA and VFA, the marginal rise of ketone bodies to 4.5 mg. per cent. was the upper normal limit for the sheep and was quite different from the 20 mg. per cent. observed in the rat (Cole, Fredericks and Engel, 1955). This is perhaps surprising because ruminants are considered to be susceptible to ketosis. The small rise in blood ketones could be related to the less extensive inhibition of acetate oxidation in sheep. The explanation of chronic poisoning is at present obscure, especially because of the occasional absence of changes in blood constituents up to the end.

The most striking change is in the citrate levels in the heart, the failure of which seems to be the usual cause of death.

CONCLUSIONS

A single dose of 0.4 mg/kg. sodium fluoroacetate was invariably fatal in sheep. Smaller doses may not produce any obvious symptoms but apparently cause some irreversible change. Further such doses are then toxic.

Changes superficially resembling "fluoroacetate diabetes" are observed in the sheep, but are probably due to sympathetic stimulation. The main biochemical effect seems to be an interference with fat utilisation, although no ketosis was observed.

Acetate had very limited prophylactic value.

ACKNOWLEDGMENTS

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AN UNUSUAL CASE OF FOWL PARALYSIS

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INTRODUCTION

The characteristic pathological feature of fowl paralysis is the macroscopically discernible enlargement, discoloration and loss of typical striation of one or more peripheral nerves. Nearly all the readily accessible nerves have been found to be affected in varying degrees and in various combinations in what must be considered typical cases. These nerve lesions are due to oedema, with infiltration by lymphocytes and plasma cells which is always prominent when the nerves are noticeably enlarged. Low-grade cellular infiltrations are sometimes difficult to find although they may be present in nerves which show no macroscopic abnormalities. Both peripheral nerve infiltrations and perivascular cuffing in the brain and spinal cord are considered to be pathognomonic; their histological demonstration is used to confirm the diagnosis of fowl paralysis.

An interesting feature of this disease is the frequent discrepancy between clinical symptoms and the distribution of macroscopic nerve lesions (Wilson, 1947). It is by no means uncommon to find no macroscopic or even histological abnormalities in the nerves of the leg in birds which show pronounced lameness. In such cases other peripheral nerves such as those of the brachial plexus or the central nervous system are affected; it is therefore not usually difficult to establish a diagnosis of fowl paralysis. Furthermore, according to Lerche and Fritzsche (1934), the clinical symptoms of such cases can often be related to spinal lesions.

On the other hand one often encounters evidence of striking correlation between local nerve lesions and functional disturbance of the organs supplied by the nerves. Dyspnoea with involvement of the vagus is quite common and for this reason gaping must be considered one of the possible symptoms of fowl paralysis. Infiltration and enlargement of the splanchnic and vagus nerves frequently lead to a characteristic dilatation of the proventriculus (Hutyra, Marek and Manninger, 1946). Infiltration of the vestibular nerve may lead to a disturbance of balance and torticolllis (Jungherr, 1952; Siller, unpublished).

The case reported here is one of megacolon, following the development of lesions typical for fowl paralysis, in the local nerve supply and CNS. Since distension of the colon is not uncommon in the fowl, it is believed that this case is of importance from the point of view of differential diagnosis.

RESULTS

Case Record

A brown leghorn pullet (4 month) in the flock of the Poultry Research Centre, Edinburgh, was killed because of a persistent watery discharge from the vent with cloacal ulceration. No nervous or other clinical symptoms had been observed. At autopsy the bird was found to be in fair bodily condition. The feathers of the vent region were soiled and the abdomen appeared to bulge posteriorly without causing a prolapse of the cloaca; the usually prominent cloacal lips appeared to be entirely absent (Fig. 1). The tissues immediately within the cloaca were necrotic. Although somewhat stenosed, the orifice was patent. The large intestine was tremendously enlarged (Fig. 1) and filled with normal faecal matter covered with excessive mucus. This enlargement commenced at the caeco-colic junction and extended caudally to the recto-cloacal junction. The remainder of the intestinal tract and the other internal organs showed no significant abnormalities. The intestinal nerve lying in the mesentery and running parallel with and dorsal to, the large intestine was somewhat enlarged. No macroscopic abnormalities were observed in any other nerves or in the central nervous system.

Histology

Brain, spinal cord, intestinal nerve and portions of the large intestine and cloaca were examined histologically. A number of blood vessels in the cerebrum, the pons, the optic lobes and particularly the optic chiasma showed varying degrees of perivascular cuffing. The Virchow-Robin spaces of some of the larger arteries of cerebrum and optic chiasma were tightly packed with lymphocytes (Figs. 2 and 3). No cerebellar lesions were found. A focal lymphocytic infiltration without apparent perivascular relationship was observed in the optic chiasma (Fig. 4). Demyelination was not found. No lesions were found in the spinal cord, although the the meninges showed areas of lymphocytic infiltration, generally with a vascular relationship.

Numerous cell aggregations, mainly lymphocytes, and generally related to blood vessels, were observed in the intestinal nerve (Fig. 5). The nerve fibres and ganglion cells appeared to be normal, without demyelination or diffuse lymphocytic infiltration.

The lymphoid nodules normally present in the submucosa of the large intestine were considerably hyperplastic, extending also into the mucosa itself. The nerve tracts of the submucous plexus of Meissner and of the myenteric plexus of Auerbach were readily discernible and were apparently normal. Heterophil leucocytes were found to be sparsely scattered throughout the mesentery of the large intestine, which did not show any macroscopic lesions.

DISCUSSION

Some consideration must be given to the diagnosis of this case. The macroscopic appearance of the large intestine brings to mind the congenital condition of the human infant known as megacolon or Hirschsprung's disease, which is characterised by a flaccid dilatation of the colon extending for a varying distance and sometimes involving even the rectum. Distally to this dilated portion is a constricted area which, according to Bodian (1949) is responsible for the development of the proximal colonic dilatation. He has demonstrated the characteristic histological lesion in Hirschsprung's disease to be absence of ganglion cells in the myenteric plexus of Auerbach. While the ganglion cells are normally distributed in the dilated segment they are absent in the distal constricted portion.

It has been pointed out above that in the present case the plexus of Auerbach and that of Meissner were both histologically normal. Ganglion cells were demonstrated not only in the dilated portion of the colon and rectum, but also distally in the region of the recto-cloacal junction. There was no constricted area comparable with that characteristic of Hirschsprung's disease.

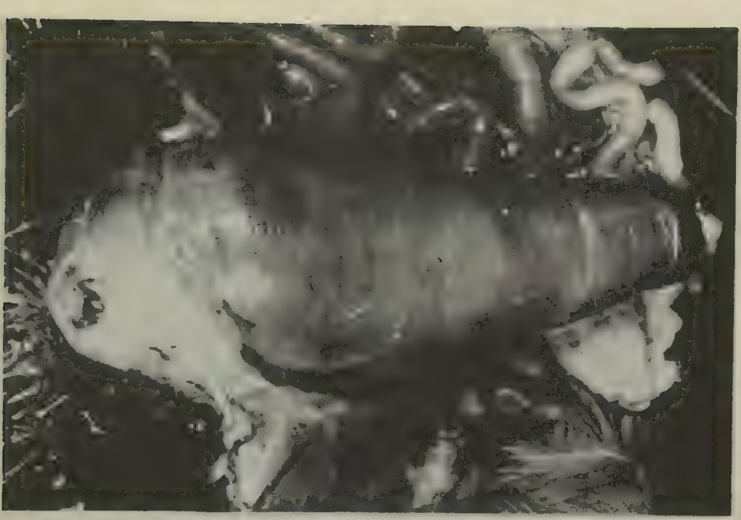
The histology of the central nervous system provides a clue to the true nature of the present case. The nervous lesions described above are similar to those observed in fowl paralysis where, according to most authorities (Lerche and Fritzsche, 1934; Wilson, 1947; Jungherr, 1952), central lesions, when present, consist of perivascular lymphocytic cuffing and sometimes more diffuse areas of lymphocytic infiltration.

Jungherr (1935) observed similar brain lesions in a comparatively large percentage of normal birds and Pappenheimer, Dunn and Cone (1929) point out that lymphoid and plasma cell infiltration in the brain and spinal cord are frequently present in fowls which showed no obvious symptoms. They interpret these as sub-clinical cases of fowl paralysis.

In the present case, foci of lymphocytic infiltration were observed in the intestinal nerve; this finding would tend to support a diagnosis of fowl paralysis. Yet Palmer and Biely (1934) described such infiltrations in the brachial plexus of apparently normal fowls and Oakberg (1950) observed similar lesions in splanchnic nerves. The latter interprets these findings as subclinical manifestations of fowl paralysis and suggests "that all extravascular lymphoid areas in nerves of chickens be considered abnormal, and as a possible indication of the presence of the causative agent of lymphomatosis." Pappenheimer *et al.* (1929) state that the lymphoid infiltrations precede myelin degeneration and that the latter therefore occurs in the more advanced cases. This would explain the absence of demyelination in the present instance.

It is believed, in view of the histological nervous lesions, that a diagnosis of fowl paralysis is justifiable in the case described above, and that a functional disturbance of the nerve supply to the large intestine was produced with a consequent loss in tonus. Wilson (1947) has drawn attention to the fact that constipation can be a symptom of fowl paralysis and interprets this as secondary to cloacal paralysis caused by the involvement of the hypogastric nerve. Tonus

Fig. 1



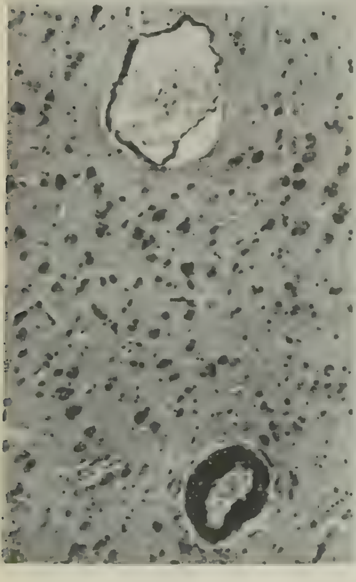


Fig. 2

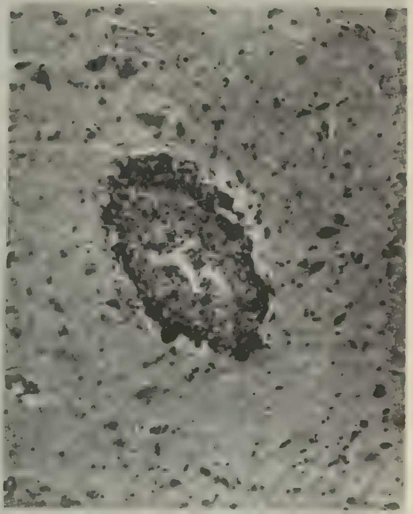


Fig. 3

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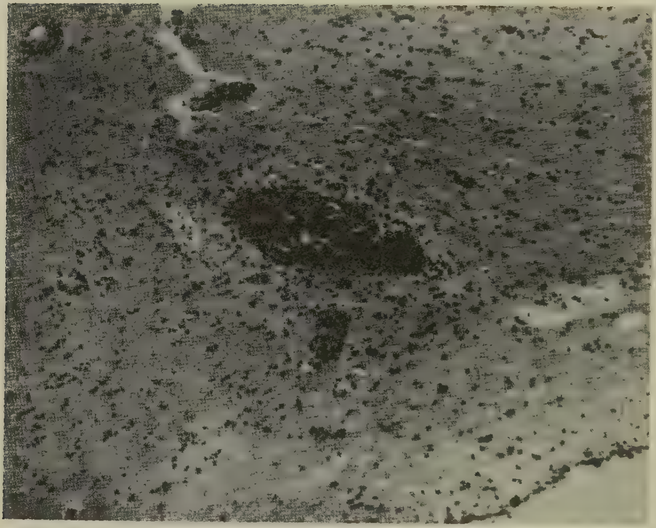
Fig. 4



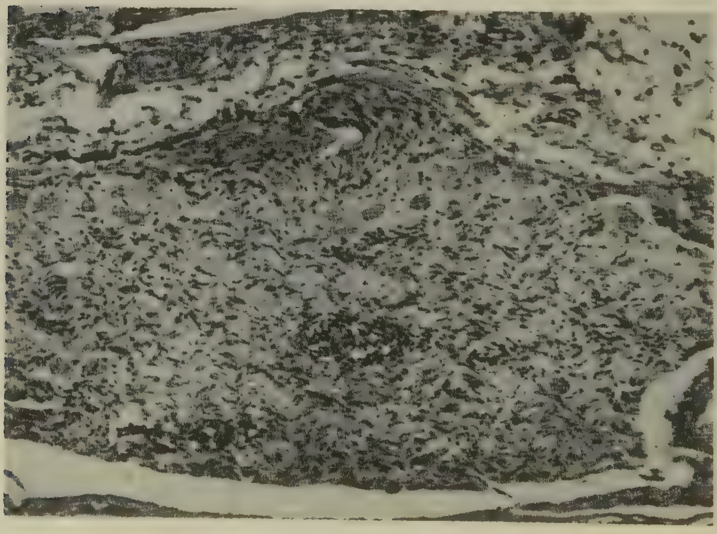


Fig. 5

in the intestine is maintained by the plexus of Auerbach. While this plexus did not show any lesions one must remember that it receives branches from the intestinal nerve which according to Hirst (1934) is unique to birds and alligators, in that it is situated in the mesentery running the whole length of the intestines and contains groups of ganglion cells along its entire length (Fig. 5). A lesion in this nerve may therefore have a direct effect on the intestinal plexuses. Simple obstruction and secondary dilatation of the large intestine would not explain the presence of nervous lesions in this case.

CONCLUSIONS

The macroscopic and histological appearance of a case of megacolon is described in a fowl which had shown no clinical nervous symptoms.

Perivascular lymphocytic cuffing in the central nervous system and lymphocyte infiltration in the intestinal nerve lead to the diagnosis of fowl paralysis.

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LEGENDS—

Fig. 1. Exposed large intestine, showing huge distension of the colon and rectum. The ulceration of the cloaca and loss of cloacal lips is illustrated.

Fig. 2. Two cerebral vessels, one showing marked perivascular cuffing. H & E × 130.

Fig. 3. Artery with perivascular cuffing in cerebrum. H & E × 130.

Fig. 4. More diffuse lymphocytic infiltrations in optic chiasma. H & E × 130.

Fig. 5. Areas of focal lymphocytic infiltration in the intestinal nerve. Note the distribution of neurons characteristic for this nerve. H & E × 130.

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CHEMOPROPHYLAXIS AGAINST BOVINE TRYPANOSOMIASIS

II. Duration of Protection afforded by Preparations of Metamidium, Prothidium and Antrycide Prosalt in an Area of High Tsetse Density

By

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INTRODUCTION

Although quinapyramine (B. vet. C. 1953) has been widely and effectively used prophylactically in the field in Africa for the control of bovine trypanosomiasis, the necessity of repeating injections every two or three months produces administrative and technical difficulties. The search for a prophylactic drug which would confer a longer duration of protection has therefore continued.

Recent investigations have been recorded by Williamson and Desowitz (1956), Robson and Cawdery (1958), Robson (1958) and Smith (1959). A drug, metamidium**, which showed promise therapeutically and prophylactically against syringe-passaged strains of *Trypanosoma congolense* in laboratory mice, has been described by Wragg, Washbourn, Brown and Hill (1958). Observations have been reported from the U.K. and from different places in Africa indicating that the drug was reasonably well tolerated by the intramuscular but not by the subcutaneous route in cattle (Lucas, 1957; Burdin, 1958; Stephen, 1958). The compound was active against field strains of species of East African trypanosomes in cattle (Fairclough, 1958).

The experiment described in this paper was carried out to obtain a measure of the average duration of protection conferred by metamidium chloride hydrochloride and its suramin salt in comparison with Prothidium† and quinapyramine (prophylactic)‡ drugs already used prophylactically in East Africa.

MATERIALS AND METHODS

Cattle. Immature, small shorthorned East African Zebu stock which had not previously been in contact with tsetse, were employed.

Drugs. These were supplied free by their respective manufacturers.

Metamidium was used as the chloride hydrochloride and as the suramin salt (Wragg *et al.* 1958) presented in one or other of two forms.

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*Formerly May & Baker Ltd., Dagenham, Essex.

**May & Baker Ltd.

†Boots Pure Drug Co. Ltd.

‡Antrycide Prosalt: Imperial Chemical (Pharmaceuticals) Ltd.

In the first, the particle size was less than three μ (M&B 4427, fine suspension); in the second, it varied from approximately 10 to 100 μ in diameter (M&B 4427, coarse suspension). Prothidium was used as the bromide salt and the quinapyramine prophylactic (B. vet. C. 1953) was the standard formulation containing 3 parts quinapyramine sulphate to 4 parts quinapyramine chloride. Each drug was made up with sterile distilled water immediately before injection, except for M&B 4427 fine suspension which was obtained from the manufacturers already suspended in distilled water; it had been prepared one month previously. The metamidium salts were injected into the muscles of the neck just behind the atlas and Prothidium and quinapyramine (prophylactic) into the dewlap. The weight of each animal was taken to the nearest five kg., and the volume of injection was adjusted to the nearest 0·5 ml.

Trypanosome challenge. The work was carried out in an area heavily infested by tsetse, predominantly *Glossina pallidipes.* Details of the experimental area and of the challenge have been given by Smith (1959) and by Smith and Rennison (in preparation). Species of trypanosomes present included *T.vivax, T.congolense,* and *T.brucei* group. The approximate infection rates of the tsetse with these species, as determined by daily dissection of samples, averaged 7 per cent, 3·5 per cent and <0·1 per cent respectively during the period of the experiment.

Measurements and recordings. Thick films of the ear blood of the cattle were made on alternate days between 7.00 a.m. and 8.30 a.m. (E. A. Standard Time) beginning four weeks before exposure to *Glossina.* After staining with Giemsa's at least 200 oil immersion fields of each film were inspected. In order to estimate the degree of swelling caused by the drug the site of injection was measured transversely with callipers immediately before and after injection and then at weekly intervals. Body weights were recorded monthly using a portable weighbridge.

TABLE I

DOSES AND NUMBERS OF ANIMALS USED

Compound	*Dose mg./kg.*		*Concentration of salt*	*No. animals exposed to challenge*		*No. animals not exposed to challenge*	*Total animals injected*
	salt	*cation*		*Male*	*Female*		
Metamidium chloride hydrochloride	4·0	3·1	4·0% soln.	10	20	4	34
M&B 4427 fine suspension	10·0	5·2	3·5% suspn.	5	10	2	17
M&B 4427 coarse suspension	10·0	4·7	3·5% suspn.	5	10	2	17
Prothidium	4·0	2·9	4·0% soln.	10	20	4	34
Quinapyramine (prophylactic)	11·7	8·1*	10% suspn.	10	20	—	30

*3·5 mg. in the sulphate salt and 4·6 mg. in the chloride salt.

Plan of investigation. The doses employed and the number of cattle in each drug group are shown in Table 1. The cattle were allotted to the groups by random process. From all the drug groups, except that treated with quinapyramine prophylactic, a number of heifers were taken at random to a tsetse free area as controls together with eight uninjected heifers. It was decided before the experiment began to analyse separately the results obtained between each pair of drugs or preparations of drugs.

After preliminary observations had been made the cattle were injected. Six days later those to be exposed to challenge were taken, together with a group of four uninjected controls, from the tsetse-free area to the experimental site. Pairs of untreated controls were introduced at intervals thereafter. When trypanosomes were detected in a film from an animal the latter was removed within 48 hours from the experimental site to the tsetse-free area.

RESULTS

Occurrence of Infection before Exposure to Challenge

No pathogenic species of trypanosome was detected in films from any animal either before injection of the drugs or before exposure to *Glossina*.

Occurrence of Infection after Exposure to Challenge

Controls. Trypanosomes (*T.vivax* and *T.brucei* group) were detected in films from the first batch of four control animals entered at the same time as the cattle injected with the drugs, from 9 to 17 days from the time they were exposed to infection. Other control groups, each comprising two animals introduced 50 and 130 days after the initial group, were also infected rapidly, the periods from the time of exposure to the detection of trypanosomes being 10 days for first pair and 6 and 12 days for the second pair. Thus the intensity of the challenge in the experimental area was high, as has been found previously (Smith, 1959). Two of the eight animals were left untreated; death occurred 10 and 20 days after parasitaemia was first seen.

Prophylactic drugs. The findings are summarised in Table 2. The mean duration of protection was significantly greater ($P < 0.01$) with M&B 4427 fine suspension than with any other drugs used, being approximately three to four weeks longer than that obtained from metamidium chloride hydrochloride and M&B 4427 coarse suspension. The latter two drugs produced approximately the same average duration of protection and there was no significant difference between them. These two metamidium preparations gave significantly longer average duration of protection ($P < 0.02$ in each case) compared with Prothidium which itself was significantly better ($P < 0.01$) than quinapyramine (prophylactic). It can be seen from Table 2 and Figure 1 that there was considerable variation in the periods before parasites were detected in the blood of the cattle in all the treated groups (cf. Davey, 1950; Fiennes, 1953;

TABLE 2

DURATION OF PROTECTION CONFERRED IN AN AREA OF HIGH TSETSE DENSITY

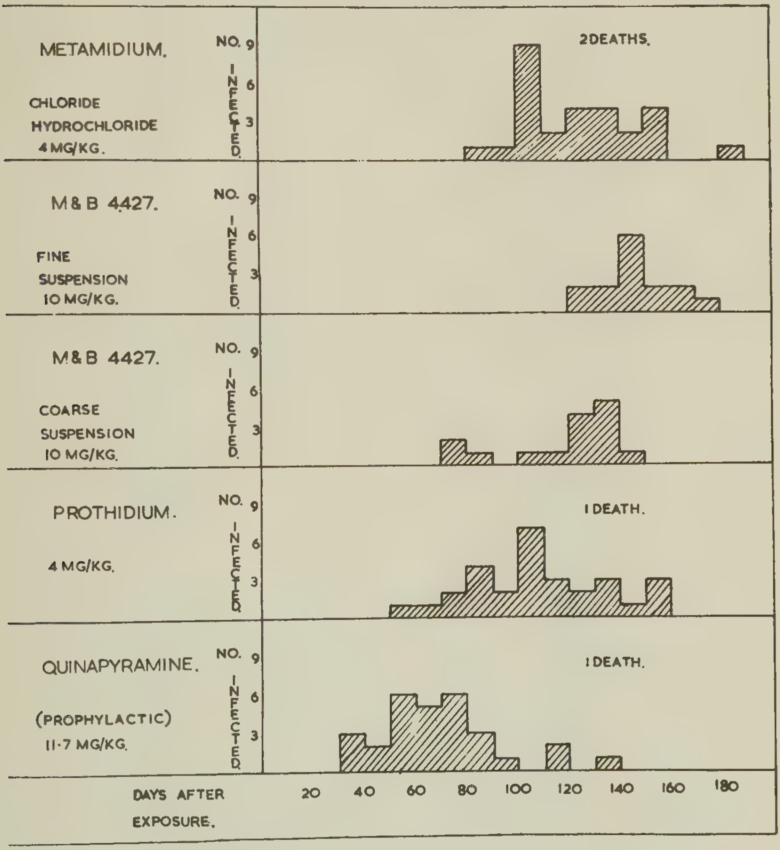
Drugs	*Dose mg./kg.*	*No. of animals in experiment*		*Approx. average period no trypanosomes detected in films weeks*	*Approx. standard deviation weeks*	*Approx. range of values recorded weeks*	*Significance*
		Beginning	*End*				
M&B 4427 Fine suspension	10	15	15	21	2	17½ – 25	Sig. better protection than other drugs below
Metamidium chloride hydrochloride	4	30	28**	18	3	12½ — 26	Not sig. different, sig. better than those below
M&B 4427 coarse suspension	10	15	15	17	3½	10 — 21	
Prothidium	4	30	29*	15½	4	8 — 22	Sig. better than quinapyramine (prophylactic)
Quinapramine (prophylactic)	11·7	30	29*	10	3	4½ — 19½	—

*Incidental death.

Smith, 1959). The occurrence of such variation makes it very difficult to select suitable regimes of prophylactic treatment in the field.

There was no significant difference in length of protection between the males and females in any of the drug groups. This finding confirms a previous observation (Smith, 1959). In considering designs of trials for testing prophylactic drugs against bovine trypanosomiasis, therefore, no attention need be paid to possible sex difference. This conclusion, however, does not eliminate the need for studies with pregnant or lactating females in which undesirable sequelae to prophylactic treatment could arise.

Fig. 1.



The distribution of the breakthroughs in each group.

The distribution of the times of breakthrough are shown in Figure 1. With metamidium chloride hydrochloride and Prothidium these times were normally distributed. For quinapyramine

TABLE 3

FREQUENCY WITH WHICH SPECIES OF TRYPANOSOMES WERE RECORDED

First positive films from cattle treated with various prophylactic drugs and exposed to a high density of tsetse

Drug	T.vivax	T.congolense	T.brucei	*Mixed infections*	*Species not recorded*
Metamidium chloride hydrochloride	12	6	3	2 *T.congolense+T.brucei* 2. *T.vivax+T.brucei*	3
M&B 4427 fine suspension	2	8	1	1 *T.vivax+T.brucei*	3
M&B 4427 coarse suspension	6	2	4	1 *T.congolense+T.brucei* 2 *T.vivax+T.congolense*	—
Prothidium	19	5	1	1 *T.vivax+T.congolense*	3
Quinapyramine (prophylactic)	16	0	3	2 *T.vivax+T.brucei* 1 *T.vivax+T.congolense*	7
Total	55	21	12	12	16

(prophylactic) the distribution was abnormal though not markedly so. The data for the two suspensions of M&B 4427 were too few for analysis. The co-efficient of variation of the breakthrough times in the M&B 4427 fine suspension group was significantly less than that of any other group, ($P < 0.05$). No significant difference could be detected between the other groups.

In previous work in the experimental area *T.vivax* was the species most commonly first detected at breakthrough (Smith, 1959). In the control animals of the experiment reported here, *T.vivax* was again the species most commonly first detected. The frequency with which each species of trypanosome was found in the dosed groups are shown in Table 3. *T.vivax* was again the most commonly recorded species except in the case of cattle treated with M&B 4427 fine suspension among which there was a relatively high proportion of *T.congolense* breakthroughs. There was a low frequency of *T.brucei* group infections (one animal) in the cattle treated with Prothidium and in those treated with quinapyramine (prophylactic) only one *T. congolense* breakthrough was noted.

It is unsafe to attempt to draw conclusions from these data because, in fact, the numbers of organisms seen at the time of breakthrough were usually very low (of the order of five trypanosomes or fewer/200 fields) and other species might well also have been present. In the cases where specific diagnosis was achieved about 12 per cent were mixed infections. However, taking the data at face value and omitting from consideration the mixed infections, a χ^2_{14} value of 46·37 was obtained. This figure indicates an association of certain drugs with certain species which is unlikely to have been due to chance ($P < 0{\cdot}01$), but it perhaps exaggerates the situation because the expected frequencies in 50 per cent of the cells were less than 5 in value, particularly in the case of *T.brucei* group infections. About two thirds of the contributions to the value of the χ^2_{14} arose from the data for M&B 4427 fine suspension, in which an unusually low frequency of *T.vivax* and an unusually high frequency of *T.congolense* obtained, and from quinapyramine (prophylactic) where an unexpectedly low frequency of *T.congolense* occurred. The latter result is not surprising; quinapyramine has long been held to be comparatively effective against *T.congolense*. The explanation of the result with M&B 4427 fine suspension is less clear, and although superficially there is a suggestion of comparatively greater efficacy against *T.vivax* and comparatively less against *T.congolense* the preparation contains the same active principle as the other metamidium salts used; with the latter the observed frequencies scarcely deviated from the expected. This result could be an indication that drug resistant *T.congolense* strains were present in the experimental area. However, of the twelve cattle where the species of parasite was identified, the eight animals infected with *T.congolense* were equally divided between the first six and the last six cattle to become positive. One would have expected the proportion of *T.congolense* infections

TABLE 4

MEAN TRANSVERSE SIZE (MM) OF SWELLINGS

Months of experiment	*Neck*			*Dewlap*	
	Metamidium chloride hydrochloride; (*34 animals*) 4 *mg./kg.*	*M&B* 4427 *fine suspension* (17 *animals*) 10 *mg./kg.*	*M&B* 4427 *coarse suspension* (*17 animals*) 10 *mg./kg.*	*Prothidium* (34 *animals*); 4 *mg./kg.*	*Quinapyramine* (*Prophylactic*) (30 *animals*) 11·7 *mg./kg.*
Immediately before injection	103	99	100	8	10
Immediately after injection	116	116	116	16	17
1	140	133	112	51	38
2	128	127	106	32	28
3	118	120	107	32	26
4	112	118	100	28	23

to have been greater in the second six animals if drug resistant *T.congolense* had been present.

It will also be observed that the number of infections with *T.congolense* was approximately half those for *T.vivax*, figures agreeing closely with the results of fly dissection studies. The number for *T.brucei*, however, greatly exceeded the expected value and if it is assumed that the probability of infection is the same for each animal, then it suggests that the *T.brucei* infection in many flies was not revealed by dissection, a point of importance entomologically and epidemiologically. It is hoped to elaborate this point in a subsequent paper.

Reaction to Injection

Systemic effects. Immediately after injection of metamidium chloride hydrochloride there was some uneasiness and excitement in the cattle, possibly attributable to pain at the site. A few minutes later when they had been released from the crush three or four of the animals showed a peculiar reaction of which the chief sign was a tendency to raise their heads, bellow, and stagger backwards in a circular direction. The response passed off in less than half an hour except in one animal, which became recumbent about four hours after injection and died eight days later. Post-mortem examination revealed that the injection of drug had been made very deeply in the neck and besides staining of the musculature covering the cervical vertebrae, the periosteum of the atlas was also coloured by the drug. In this animal, therefore, it was possible that some of the solution had penetrated into the spinal canal though no direct evidence of this was found. Systemic effects were not observed to occur with any of the other drugs.

Local reaction. With metamidium chloride hydrochloride a swelling developed within 24 hours of injection. The reaction steadily increased in size. It remained prominent for about six weeks and then slowly regressed, being still palpable three to four months after the injection had been given (Table 4). For the first six weeks or so it was apparently slightly painful, but it did not appear to interfere to any extent with the mobility of the head or neck or with grazing. M&B 4427 coarse suspension produced a smilar type of response though to a less degree. The swellings did not appear to be painful and had almost disappeared after about the sixth week, though a small indurated area round the point of injection was still palpable some four months later. The fine suspension of M&B 4427 produced a reaction very much resembling in extent and duration that from metamidium chloride hydrochloride. In five animals, however, the reaction was particularly severe and greater than in any animal injected with metamidium chloride hydrochloride. There remained six months after administration a still prominent, slowly resolving but not obviously painful swelling into which it would have been difficult to reinject. Bacteriological examination made on the material for injection before use did not reveal any pathogens likely to have caused the response and since a fresh sterile needle was used for each animal, it seems unlikely that these five severe reactions could be attributed to bacterial contamination, and they were presumably an effect of the drug itself.

The reaction in the dewlap to Prothidium was similar to that which has been already described for Zebu cattle in East Africa (Robson and Cawdery, 1958; Smith, 1959). Quinapyramine (prophylactic) gave the usual response.

Other Measurements

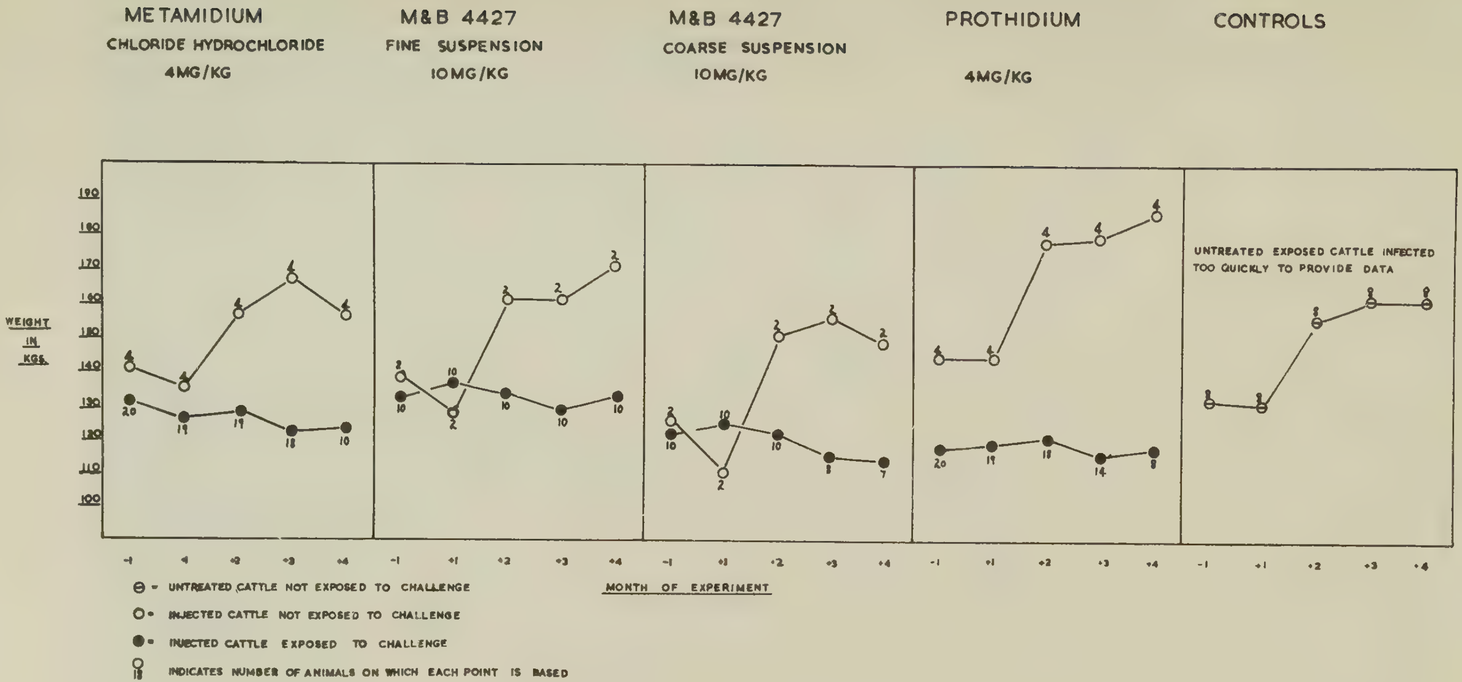
Body weights. In the cattle exposed to challenge there was little tendency to gain weight thus contrasting sharply with their homologous controls not exposed to challenge, which gained weight at a rate comparable with the uninjected control group also not exposed to challenge (Fig. 2). The results for these latter categories, based as they are on small numbers of animals, cannot give reliable indications of trends, but it seems reasonable to accept that none of the drugs produced any local or systemic effect which grossly interfered with general well being. The failure of the cattle exposed to challenge to make comparable gains is not easy to explain, but a possible reason may have been that during daylight the annoyance caused by tsetse interfered seriously with feeding and rumination; the animals ruminated only at night. There did not appear to be any marked difference in the quality of the grazing in the experimental and tsetse free areas as most species of grass were common to the two areas. Indeed, the grasses in the former appeared to be in better condition.

DISCUSSION

Whiteside (1955) showed that the intensity of the trypanosome challenge plays an important part in determining the frequency with which quinapyramine (prophylactic) needs to be given to obtain complete continuous protection in the cattle population at large. Our work was carried out in an area of comparatively very high challenge and, if the relationship between trypanosome challenge and effectiveness holds for other drugs, it is possible that longer average protection would be obtained with the drugs described in this paper in places where the challenge is less severe.

The clear superiority of M&B 4427 fine suspension over the other preparations of metamidium may have been due entirely to the greater amount of active cation administered. However, the duration of protection obtained with metamidium chloride hydrochloride was slightly longer, though not significantly so, than that from M&B 4427 coarse suspension, although in the latter case a larger amount of cation was given. The difference, therefore, seems to be too great to be explained entirely on the basis of the amount of cation administered. These findings with the fine preparation focus

Fig. 2.



METAMIDIUM CHLORIDE HYDROCHLORIDE 4MG/KG

M&B 4427 FINE SUSPENSION 10MG/KG

M&B 4427 COARSE SUSPENSION 10MG/KG

PROTHIDIUM 4MG/KG

CONTROLS

Body weight changes in each group.

attention on a point to which probably insufficient consideration has previously been given in prophylaxis against bovine trypanosomiasis, namely, the possible association between the length of protection obtained and the physical state of the material injected. In other fields particle size in the injection has been found to be important, for example, in phenothiazine therapy (Gordon, 1956) and in insulin therapy (Fishburn, 1956).

Much stress has always been laid on the necessity for a depot of a drug in chemoprophylaxis against bovine trypanosomiasis. The lengthy protection given by metamidium chloride hydrochloride, a highly water soluble compound, might appear surprising, but its fate in the body may be similar to that of other soluble drugs e.g. pentamidine and stilbamidine, which exert a prophylactic effect. Ormerod (1958) has found that stilbamidine is retained by the body cells and subsequently slowly released by them and Fulton and Mathews (1959) have detected it in the liver in an unconverted state some time after dosing. Metamidium shares with these compounds the presence of an amidine group which has been shown to be associated with prophylactic activity in the diazoaminophenylphenanthridine series, of which metamidium is a member (unpublished data). Some of our observations (unpublished data) indicated, however, a deposition from solution of metamidium chloride hydrochloride in the cervical musculature, thus possibly producing the depot effect. The study of this reaction is still in progress. In any event elaboration and test of other salts and preparations of metamidium seems desirable in an effort to obtain a duration of protection approximating to that produced by M&B 4427 fine suspension.

To be acceptable for field use a prophylactic drug must be reasonably well tolerated systemically and locally. The adverse environmental conditions of many African cattle and their poor physical state require that any drug for prophylaxis against trypanosomiasis be tested widely before it is used on a large scale. Our data on body weights, in as far as such a measurement is capable of demonstrating the occurrence of any toxicity, indicated that on the whole none of the drugs produced any gross systemic ill effects, either immediate or delayed, and the cattle remained in reasonably good condition. The failure of those exposed to challenge to gain weight has been mentioned and a possible explanation offered. The local reaction at the site of injection is important, but a satisfactory, objective method for its description and measurement has yet to be established. Estimates of swelling in one plane, such as we made, take no account of the full extent of the lesion, and clearly for complete description other planes must also be measured. The longer the interval in the field between necessary injections the more severe can the lesion be, provided that stock owners are willing to accept it, and the reaction does not interfere with injection or absorption of subsequent doses. The general physical state must also be unimpaired. The reactions to metamidium chloride hydrochloride and M&B

4427 coarse suspension appeared acceptable when considered in conjunction with the average duration of protection obtained.

Five points may be greatly relevant in interpreting the results of comparative drug trials conducted in a natural challenge. Firstly, drug resistant trypanosomes present in the area before an experiment begins may be transmitted to the cattle by the tsetse population. Secondly, the work of Whiteside and Lyttle (1957) indicates that a considerable degree of cross resistance to antitrypanosomal drugs appears to occur, even when the latter are not particularly closely related chemically, and it is possible that resistant trypanosomes appearing in cattle given a less effective prophylactic drug may be transmitted by vectors during the experiment to animals treated with a possibly more effective agent. If there is cross resistance between the two drugs then the average duration of protection in the second group of animals may appear to be considerably shorter than it might have been had cattle injected with the less effective compound not been present. Thirdly, in attempting to avoid the possibility of trypanosomes, perhaps drug resistant, being picked up from the experimental cattle by vectors, we resorted to removal of animals almost immediately after they were first found to have a parasitaemia. This procedure may have been ineffective, for it may be presumed that protection must have failed at some time before trypanosomes were found in the blood films. Since the presence of the latter is not a particularly efficient method of detecting infection it seems reasonable to assume that tsetse might have been infected with trypanosomes which could have been drug resistant. Apart from its great importance generally the question of possible drug resistance of trypanosomes requires detailed investigation even in the more restricted aspect of the interpretation of comparative drug trials. Moreover, removal of infected cattle may result in greater numbers of tsetse and other biting flies feeding on the remainder thus enhancing the possibility of both infection and transmission.

Because of these three possible sources of bias it does not follow that the results for one drug are independent of the others, and it is clear that caution is required in the interpretation of findings. If, however, one agent is found to be significantly better than another, this would seem a clear indication of its superiority because the biases tend to produce their effect in the opposite direction.

A fourth possible bias which may operate in either direction is variation in challenge. If one of the drugs is greatly superior to the standard, the challenge may alter after most of the animals treated with the standard compound have been removed. Under conditions of heavy challenge the use of untreated controls may be too insensitive to detect other than major changes. Fifthly, immunological responses may be involved in prophylaxis against bovine trypanosomiasis in the field, though their role is ill understood (Fiennes, 1953; Soltys, 1955).

The factors mentioned above do not appear to have seriously influenced our results. The average duration of protection obtained with Prothidium and quinapyramine (prophylactic) was roughly similar to that experienced previously in experiments in the same area (Smith, 1959) in the face of a challenge not noticeably less severe than formerly (unpublished data). The curves of the times of breakthrough for the metamidium compounds do not suggest that drug resistant trypanosomes were passing from, for example, the animals treated with metamidium chloride hydrochloride to those injected with M&B 4427 fine suspension. Nevertheless the comparatively very short intervals between the times of breakthrough for the first and last animals, particularly in the group given M&B 4427 fine suspension, is suspicious, even although it may be explicable on other grounds, e.g. increasing challenge as the number of animals present progressively decreased, or alternatively on the physico-chemical properties of the drug. The unexpectedly high proportion of breakthroughs with *T. congolense* in this drug group has already been mentioned. Tests of strains recovered from the cattle in this experiment are in hand and it is hoped to record the results in a subsequent paper.

From what has been said of possible sources of bias it will be realised that the investigation of the comparative efficacy of prophylactic drugs presents difficulties which are not easily overcome. Drugs can be compared on a small scale by the syringe inoculation of trypanosomes but the findings have to be interpreted cautiously because of the effects of syringe passage on the organism and its general lability. Cyclical challenge in the laboratory is hampered by the fact that only comparatively small numbers of cattle can be dealt with. Since the variation in the duration of protection from a single dose of a prophylactic drug is very great (Davey, 1950; Fiennes, 1953; Robson and Cawdery, 1958; Smith, 1959) comparatively large numbers are needed to give reasonable estimates for determining the intervals between doses in regime trials. Obviously further attention should be paid to improving the type of design employed to try to overcome the possible sources of bias which are inherent in the present type of trial.

CONCLUSIONS

An experiment was conducted in an area densely infested with tsetse, chiefly *G.pallidipes,* to compare the duration of protection against trypanosomiasis conferred on Zebu cattle by metamidium salts, Prothidium and quinapyramine (prophylactic).

The approximate average durations of protection produced were: M&B 4427 fine suspension (10 mg./kg.), 21 weeks; metamidium chloride hydrochloride (4 mg./kg.), 18 weeks; M&B 4427 coarse suspension (10 mg./kg.), 17 weeks; Prothidium (4 mg./kg.), 15½ weeks; quinapyramine (prophylactic) (11·7 mg./kg.), 10 weeks.

Slight systemic effects, which rapidly passed off, were observed with metamidium chloride hydrochloride immediately after injection. Clinical appearances and body weights did not suggest the occurrence of any delayed systemic effects in the cattle. With the preparations of metamidium the local reaction at the site of injection intramuscularly in the neck was worse than that observed with Prothidium and quinapyramine (prophylactic) subcutaneously in the dewlap.

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UGANDA ZEBU CATTLE NATURALLY INFECTED WITH *FASCIOLA GIGANTICA* WITH SPECIAL REFERENCE TO CHANGES IN THE SERUM PROTEINS

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INTRODUCTION

The greater part of the published work dealing with the effects of fluke infection in cattle concerns the infection of European cattle by *Fasciola hepatica*. The majority of the statements concerning *Fasciola gigantica* appear to have been derived by analogy with the effects seen in cases of *F. hepatica* infection rather than from direct observation.

In most parts of Uganda *F. gigantica* is enzootic and the parasite load in the cattle is usually heavy. The observations described here constitute part of an investigation to assess the pathogenic effects of *F. gigantica* in indigenous Zebu cattle.

MATERIALS AND METHODS

Experimental animals. The infected group consisted of twenty animals purchased in an enzootic area. At the time of purchase they were all in poor condition; animals offered for sale with overt signs of some other disease were rejected. A control group of ten animals was selected from the Zebu stock available at the Animal Health Research Centre, Entebbe. They were presumed to have had no contact with *F. gigantica* as all watering is from troughs and no *Lymnaea* species have been found on the station at any time during the last ten years. During the experiment, conditions of husbandry were identical for both groups and all investigations were carried out in parallel on both groups with the exception of post mortem findings, as there seemed little point in slaughtering valuable healthy animals.

Plan of experiment. The investigations were divided into two sections, parasitological and biochemical. To supplement the findings liveweights were recorded and the general condition of the animal was assessed and was classified as "good", "fair", "poor", or "bad". At the end of the observation period all the infected cattle were slaughtered and the number of *F. gigantica* in each liver was counted. A naked eye estimation of the degree of liver damage was also made. Any other abnormalities in the carcase, and parasites other then *F. gigantica*, were recorded.

Parasitology. The nature and extent of the parasite burden were assessed by examination of faeces and blood. Estimation of the trematode ova in the faeces was done by the sedimentation method described by Coyle (1958) while the McMaster method, modified by centrifugation

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TABLE 1

DATA FOR INFECTED SERIES AT SLAUGHTER AND LIVEWEIGHTS OF CONTROL GROUP

Animal number	*Severity of liver damage*	*Number of flukes found in liver*	F. gigantica *ova (ova/g. faeces)*	*General condition*	*Liveweight (lb.)*	*Liveweight (lb.) Infected group*		*Liveweight (lb.) Control group*	
6023	++++	310	26·8	Moribund	315	Mean	368	Mean	563
6013	++++	364	7·4	Bad	359	Maximum	433	Maximum	708
6015	++++	200	29·4	Bad	305	Minimum	305	Minimum	540
6018	++++	6	Nil	Bad	375				
6028	++++	Nil	Nil	Bad	316				
6009	+++	62	3·4	Fair	400				
6030	+++	60	3·2	Poor	389				
6019	+++	45	0·5	Poor	372				
6012	+++	15	11·8	Bad	321				
6024	+++	10	2·4	Bad	344				
6029	+++	1	0·1	Bad	433				
6026	++	45	1·9	Poor	390				
6027	++	42	1·7	Bad	400				
6008	++	4	Nil	Fair	430				
6017	++	2	Nil	Fair	325				
6011	++	Nil	0·8	Poor	356				
6025	+	20	3·4	Fair	400				
6021	+	10	Nil	Fair	405				
6020	+	6	Nil	Poor	310				
6016	+	Nil	Nil	Fair	384				

at 1500 r.p.m. for five minutes, was used for nematode ova. Blood smears were made at intervals of three days throughout the observation period and were examined for protozoan parasites, but none was seen.

Biochemistry. The estimations described in this section were carried out on serum samples obtained from jugular blood. The total serum protein was estimated by the biuret method as described by King and Wootton (1956). Paper electrophoresis was carried out according to the method of Flynn and de Mayo (1951) using a pH 8·6 veronal-acetate buffer of ionic strength 0·1. A quantity of 0·02 ml of serum was applied to a strip of Whatman Number 1 paper 36 cm by 5 cm in size and a current of 1·5 mA was applied to each strip for eighteen hours. The paper strips were stained with bromophenol blue, soaked in liquid paraffin, and scanned with an automatic scanner (E.E.L.)*.

The results were subjected to a statistical analysis using the entire group of twenty infected animals and also, because one was moribund and had a total serum protein well below the expected range, for the remaining nineteen as a group. The exclusion of this animal made little difference to the statistical findings. A difference equalling two standard errors between sample means is regarded as being probably significant. If the difference between the sample means exceeds three standard errors it is regarded as being highly significant.

RESULTS

The information in Table I incorporates the parasitological findings, the assessment of the condition of the animals prior to slaughter and the post mortem findings in the liver. For simplicity of presentation the post mortem findings are represented by signs; one plus sign indicates moderate liver damage and increasing damage is denoted by the addition of extra signs up to a maximum of four. This table shows that most of the infected animals were in poor or bad condition just before slaughter and one was moribund. The liveweights were low compared with those for the group of control animals and despite good husbandry during the period of observation the infected animals suffered a steady decline in condition. The fact that infection by *F. gigantica* causes loss of condition in cattle has also been shown by Alicata, Swanson and Goo (1940) working in Hawaii.

Eleven of the animals had less than twenty flukes in the liver at slaughter but some of these showed as severe liver damage as those with a heavy infection. *F. gigantica* ova were demontrated in the faeces of all the infected animals at some time during the observation period, but never in the controls. In the case of two animals there were neither ova in the faeces nor flukes in the liver at slaughter; however, severe liver damage had occurred, probably as the result of recent infection. Paramphistomes and nematodes were found in all the infected animals, but at no time did the nematode burden reach likely pathogenic proportions, while paramphistome infection is regarded as insignificant in adult animals.

The data obtained from electrophoretic separation of the serum

TABLE 2

SERUM PROTEIN CHANGES

	Albumin		*α Globulin*		*β Globulin*		*γ Globulin*		*Albumin/Globulin ratio*		*Total protein g.%*	
	Controls	*Infected*	*Controls*	*Infected*	*Controls*	*Infected*	*Controls*	*Infected*	*Controls*	*Infected*	*Controls*	*Infected*
Mean	34·9%	28·5%	17·1%	14·3%	19·5%	17·6%	28·4%	39·6%	0·550	0·400	7·25	7·45
Maximum ..	41·5%	37·6%	19·8%	24·3%	23·9%	25·2%	32·9%	48·6%	0·710	0·600	8·4	8·6
Minimum ..	28·8%	15·3%	15·3%	8·9%	14·8%	13·2%	23·8%	32·8%	0·410	0·180	6·3	5·1
Standard deviation	3·70	5·60	1·30	3·41	2·40	3·21	3·00	3·50	0·089	0·107	0·627	0·775
Difference of means	6·40		2·80		1·90		11·20		0·150		0·200	
Standard error of difference x 3	3·90		2·58		3·09		3·67		0·111		0·795	
Significance ..	Highly		Probably		Not		Highly		Highly		Not	

protein fractions of both series and the values for the total serum proteins are given in Table 2. There is a statistically significant difference between the values for albumin, α globulin, γ globulin and the albumin/globulin ratio irrespective of whether the series contains the moribund animal or not. There is no significant change in the values for β globulin or total protein between the control and infected groups.

DISCUSSION

According to Marrack and Hoch (1949) the change in serum proteins characteristic of disease of the liver parenchyma in man is increase of γ globulin while the albumin is reduced, sometimes to very low levels, and the other fractions are usually little changed. That these changes occur in cattle has been shown by Campbell (1957) who reported an increase in γ globulin, a fall in albumin, and inconstant small changes in the α and β fractions in bovine hepatic cirrhosis. In the present series of Zebu cattle, considerable liver damage was demonstrated at post mortem examination and this is reflected in the changes in the serum protein fractions. Similar changes in the protein fractions were reported by Komine, Usui, Watanabe and Sugiura (1955) in the sera of Japanese cattle infected with *F. hepatica*. These workers also showed, as do the results in the present series, that there was no significant change in the total serum protein in cases of fluke infection.

In this present series the serum α globulin of the infected group is lower than that for the control group. The difference between the means exceeds three times the standard error, but the difference is small. This difference is regarded as significant and in any case exceeds the range of small changes reported by other workers.

CONCLUSIONS

Total serum proteins were estimated and paper electrophoretic separation of the serum protein fractions effected for a group of Zebu cattle naturally infected with *F. gigantica*. These values were compared with those obtained for a group of control animals. Faecal ovum counts were done on the infected animals and a post mortem examination carried out, with especial regard for the degreee of liver damage and the number of flukes found in the liver.

It was shown that severe irreversible liver damage, which was not necessarily associated with a large number of flukes in the liver at the time of post mortem examination can result from infection with *F. gigantica*.

There was a highly significant increase in γ globulin with a fall in albumin in the group of infected animals, a finding which is consistent with a considerable degree of liver damage.

The general condition of the infected animals was strikingly poor and deteriorated throughout the period of observation.

These findings provide further evidence that *F. gigantica* infection in cattle in the enzootic areas of Uganda constitutes a serious disease problem.

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FURTHER OBSERVATIONS ON THE EXPERIMENTAL PRODUCTION OF SCRAPIE IN GOATS AND SHEEP

By

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INTRODUCTION

Pattison, Gordon and Millson (1959) reported the experimental production of scrapie in goats, and drew attention to the high take in this species—compared with sheep—following intracerebral inoculation of scrapie brain suspension or of cerebrospinal fluid (C.S.F.) from scrapie-affected animals.

The present paper reports the results of further observations on the experimental production of scrapie in goats and sheep.

MATERIALS AND METHODS

Goats. Some of the goats used were from the Compton herd of cross-bred animals, but others were obtained as kids, of various breeds, from a wide variety of sources. The sex and age of animals in the various experiments are given under "Results".

Sheep. The sheep used were from the Compton flock in which naturally occurring scrapie has not been recorded.

Scrapie materials. Unless otherwise stated, the "strain" of scrapie used was that described by Pattison *et al.* (1959).

Inocula. Inocula were prepared from various body tissues by grinding each tissue in a mortar with sterile sand, adding physiological saline to make a 10 per cent w/v suspension and centrifuging at about 1,500 r.p.m. for 15 minutes. The supernatant formed the inoculum.

RESULTS

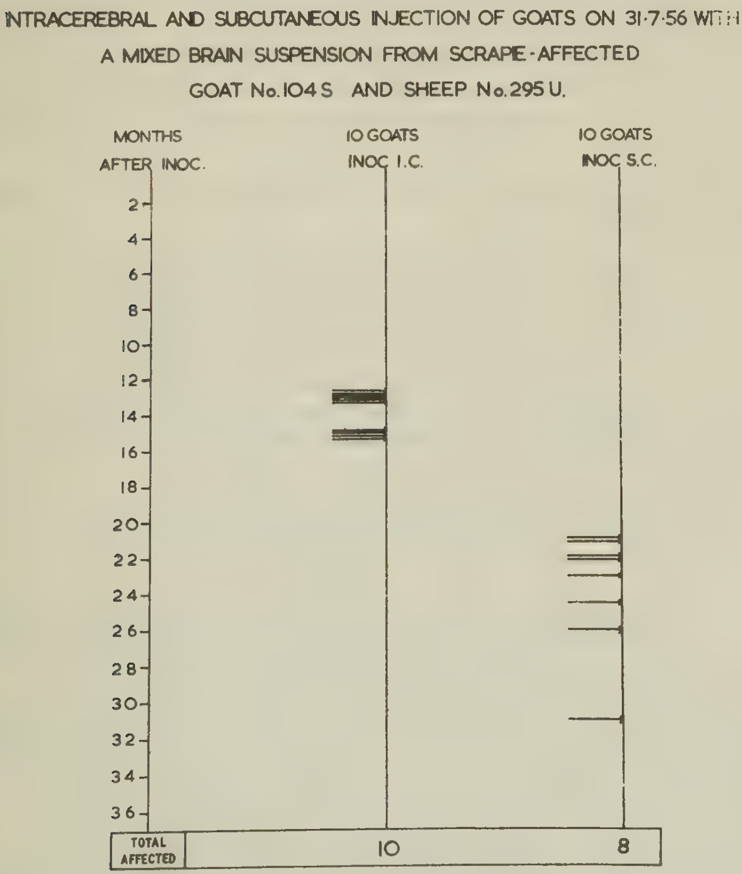
Further Intracerebral Passage of Scrapie through Goats

Pattison *et al.* (1959) reported that inoculation of 1·0 ml. of scrapie brain suspension intracerebrally produced the disease in all of 50 goats, through four passages. One further passage has now been made, and again the disease has been produced in every goat. The inoculated animals were six castrated males about eight weeks old. The inoculum used was a suspension of the pooled brains from two goats of the fourth passage. The disease first appeared eight months after inoculation and all the animals were affected within 11 months. In this passage the first three cases developed quickly, with marked head tremors; in one case the time from onset to the terminal stage was only three weeks, compared with the usual course of three to five months. In the other three animals the usual more protracted course was observed. It would appear that in passages of the disease through goats, using brain suspension inoculated by the intracerebral route, the incubation period is about eight to twelve months, compared with the original incubation period (Pattison *et al.* 1959) of 15 to 22 months when the disease was first produced in goats using scrapie sheep brain as the inoculum.

Comparison of the Intracerebral and Subcutaneous Routes in producing Scrapie in Goats

In order to determine whether scrapie could be produced in the goat, as in sheep (Gordon, 1957) by subcutaneous inoculation and to compare this with the intracerebral route, twenty female goats about five months old were randomly divided into equal groups and inoculated on the same day either with 1·0 ml. of brain suspension intracerebrally or with 5·0 ml. of the same suspension subcutaneously behind the shoulder. The brain suspension used was

Fig. 1.



Development of scrapie in goats inoculated intracerebrally or subcutaneously with scrapie brain suspension.

prepared from approximately equal amounts of a scrapie goat brain held for six months in deep freeze and of a scrapie Cheviot sheep brain held for five days in deep freeze. The reason for using a mixture of scrapie sheep brain and scrapie goat brain for these inoculations was that the ability of scrapie goat brain to reproduce the disease had not at that time (1956) been established. A young, non-inoculated male goat was put with these females, most of which kidded during the period of observation.

The results of this experiment are given in Figure 1, from which it will be seen that scrapie occurred in all the intracerebrally inoculated animals between 12 and 15 months after injection, whereas the first case in the subcutaneous group did not appear until 21 months after injection. At the time of writing (34 months after inoculation) the disease has occurred in eight of the ten animals inoculated subcutaneously; observation of the two remaining animals continues. It would appear, therefore, that there is a significantly longer period of incubation in goats inoculated subcutaneously than in those inoculated intracerebrally with the same material. In individual experiments, this lengthened incubation period has also been observed in sheep inoculated subcutaneously (Gordon, 1957).

Scrapie in Goats inoculated Subcutaneously with large Doses of Scrapie Brain Suspension

The long period that elapses between a single injection of scrapie brain suspension and the appearance of clinical disease in goats makes experimental work difficult and expensive. Any method that will shorten this incubation period would be of considerable practical importance. For this reason it was considered worthwhile to examine the effect of multiple subcutaneous inoculations of scrapie goat brain suspension, the hope being that injection of these large amounts might significantly reduce the incubation period.

Six eight-week-old animals were used, four castrated males and two females. Four were injected with scrapie goat brain suspension and two with normal goat brain suspension. Two of the animals inoculated with scrapie brain received subcutaneous injections of 10 ml. on 50 consecutive days, and the other two received 10 ml. on 30 consecutive days. The two animals given normal brain suspension received 10 ml. on 30 consecutive days. The scrapie-producing potential of the brain suspensions used was checked by intracerebral inoculation of other goats with 1·0 ml. of the suspension used for the last subcutaneous inoculation in each series. The scrapie brain suspension produced the disease, after an incubation period of approximately 12 months, in all of six animals inoculated intracerebrally. The normal brain suspension did not produce the disease in either of two animals inoculated intracerebrally and kept under observation for two years.

All the four goats given large amounts of scrapie goat brain subcutaneously developed the disease, two at 11 months after the last injection, one at 12 months and one at 13 months. The two animals given large doses of normal brain subcutaneously showed no evidence of disease within an observation period of two years after the last injection. Thus, the incubation period of about 12 months following multiple large subcutaneous injections of scrapie brain suspension was the same as has been observed in many goats given a single 1·0 ml. intracerebral injection of brain suspension and significantly shorter than when a single subcutaneous injection was given (Fig. 1).

Intracerebral Inoculation of Goats with Scrapie Goat Brain previously treated with Ribonuclease or Deoxyribinoculease

Dr. R. Markham of the Agricultural Research Council Virus Research Unit, Cambridge, kindly carried out the enzyme treatment of scrapie goat brain suspension. A 10 per cent w/v suspension of scrapie goat brain in physiological saline was prepared in the usual manner and 1·0 ml. of this was incubated for two hours with 70 μg. of ribonuclease; another 1·0 ml. amount was incubated for two hours with 100 μg. of deoxyribonuclease. These prepearations were then held at −18°C. for 27 days prior to intracerebral inoculation of goats. The animals used were castrated males about four weeks old. Two animals received 0·5 ml. each of the ribonuclease-treated material, and two animals received 0·5 ml. each of the deoxyribonuclease-treated material. Four other animals were given 0·5 ml. intracerebrally of the untreated brain suspension, also held at −18°C. for 27 days.

Symptoms of scrapie were first observed about nine months after inoculation and all eight animals were affected one month later. There was no difference in the incubation period between animals inoculated with the enzyme-treated or with the untreated brain suspension. It was concluded, therefore, that this enzyme treatment had not detectably altered the scrapie-producing potential of the scrapie brain suspension.

Resistance of the Scrapie-producing Agent in homogenized Scrapie Sheep Brain to Transport from Britain to Canada and back to Britain

In order to check the infectivity of scrapie sheep brain sent from Moredun Institute, Scotland, to Canada for inoculation of animals in that country, the material that remained after these inoculations had been made was returned to Compton for testing. The material received at Compton consisted of 8·7 g. of homogenized scrapie sheep brain, and 42 days had elapsed since its dispatch from Moredun to Canada. Sterile physiological saline was added to the brain paste to make a 10 per cent w/v suspension which was then centrifuged for about 10 minutes at 1,500 r.p.m. Each of 30 South

Scotland Cheviot wethers about 15 months old and three castrated male goats about 3 months old was inoculated intracerebrally with 1·0 ml. of the supernatant from this centrifuged suspension.

Twelve cases of scrapie occurred among the 30 sheep, from five to nine months after inoculation and observation of these animals was discontinued after 11 months. The first goat showed clinical evidence of scrapie 18 months after inoculation and all three were affected by 19 months.

It would appear, therefore, that the scrapie-producing agent shows considerable resistance to those fluctuations in temperature likely to be encountered during transit through ordinary mail. It may also be noted that the incubation period of about 19 months recorded for these three goats was similar to that recorded by Pattison *et al.* (1959) for other goats inoculated with scrapie sheep brain, and that it was significantly longer than is usually observed in goats inoculated intracerebrally with scrapie goat brain. A point of practical significance was that all of the three inoculated goats developed the disease, whereas only 12 cases occurred in the 30 sheep. Thus, the objective of the experiment—to demonstrate that the material received back from Canada was capable of producing the disease—could have been achieved by the use of a small number of goats only. This added confirmation of the high degree of susceptibility of the goat to intracerebral inoculation with scrapie brain suspension has now made it possible to reduce considerably the numbers of animals used in scrapie experiments.

Rectal Temperature of Goats during the Incubative and Clinical Stages of Scrapie.

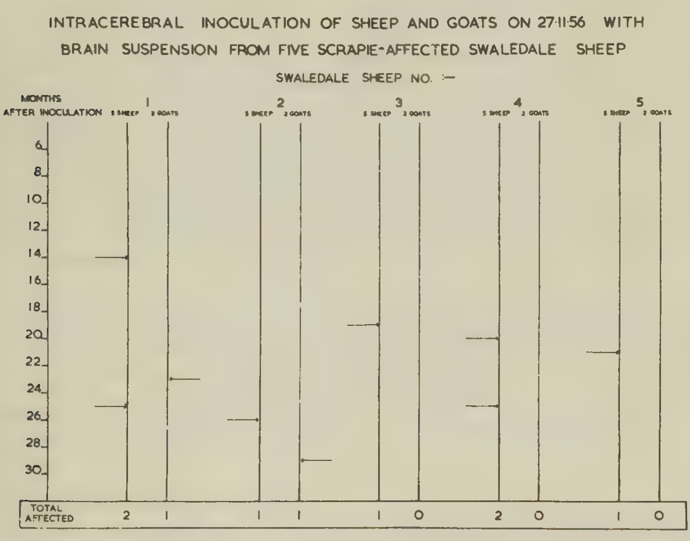
In order to determine whether any deviation from the normal rectal temperature occurs during the incubative and clinical stages of scrapie in goats, twice-daily temperature recordings were made on two goats inoculated with scrapie goat brain suspension from five days prior to inoculation until they were in an advanced stage of the disease 382 days and 452 days later, respectively. Twice-daily temperature recordings were also made over a similar period on two goats inoculated with normal goat brain suspension. The temperatures of all four animals remained within the normal range over the experimental period and there was no indication that the development of scrapie was associated with any deviation of the rectal temperature from normal.

Scrapie in Sheep and Goats inoculated Intracerebrally with Brain Suspension from Swaledale Sheep affected with naturally-occurring Scrapie

In almost all British work on the experimental transmission of scrapie, the disease-producing material used has been obtained originally from field cases of scrapie in the Cheviot breed. It appeared desirable, therefore, to determine whether the disease could be experimentally induced with material obtained from naturally-affected animals of another breed.

Five young Cheviot wethers and two female goats about eight months old were inoculated intracerebrally with 1·0 ml. of a 10 per cent suspension of brain obtained from each of five Swaledale ewes naturally affected with scrapie; these animals were obtained from different farms and showed the characteristic clinical symptoms of the disease. Details of the occurrence of scrapie in the inoculated animals are given in Figure 2, from which it will be seen that one or more cases of scrapie occurred in each group of inoculated animals, and that to the time of writing, 30 months after inoculation, seven of 25 sheep and two of ten goats have developed the disease. The unusually long delay between inoculation and the appearance of clinical symptoms in both sheep and goats in this experiment is of interest. Observation of the remaining goats is being continued.

Fig. 2.



Development of scrapie in Cheviot sheep and goats inoculated intracerebrally with brain suspensions from scrapie-affected Swaledale sheep.

Scrapie in Goats inoculated Intracerebrally with various Body Tissues

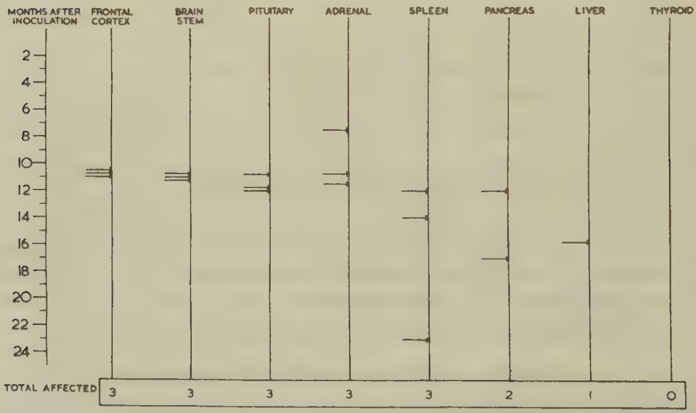
Pattison *et al.* (1959) reported the experimental production of scrapie in goats following intracerebral inoculation of scrapie brain suspension, or of cerebrospinal fluid taken from goats in the clinical stage of the disease. Gordon (1957) recorded that, in addition to various C.N.S. suspensions, a 1:5 suspension of spleen from scrapie-affected sheep produced the disease in five of 20 sheep inoculated subcutaneously with 5·0 ml. The scrapie-producing potential of various other tissues has now been examined, and the results of these inoculations were as follows:

Cerebrospinal fluid. The ability of C.S.F. from scrapie-affected goats to produce scrapie has been confirmed by injecting three four-month-old castrated male goats intracerebrally with 1·0 ml. The disease occurred in two of these animals 12 months after inoculation, and in the third animal 15 months after inoculation.

It has been noted that C.S.F. is easily obtained in considerable quantity, 20 ml., or more, from all scrapie-affected goats by puncture through the cisterna magna, whereas it can only be obtained from normal goats with some difficulty and in much smaller amount. This finding suggests that there is an increase in quantity of C.S.F. in scrapie-affected animals. However, no deviation from the normal water-clear appearance of the C.S.F. has been observed in scrapie-affected animals.

Various body tissues. In order to examine the ability of body tissues other than central nervous system to produce scrapie, castrated male goats about 12 weeks old were inoculated intracerebrally with 1·0 ml. of various tissue suspensions from a scrapie-affected goat, as shown in Figure 3. An equal number of goats was inoculated intracerebrally with the same volume of suspensions of the same tissues taken from a clinically normal goat.

Fig. 3.


INTRACEREBRAL INOCULATION OF GOATS ON 23·5·57 WITH VARIOUS TISSUES FROM A SCRAPIE-AFFECTED GOAT

3 GOATS INOCULATED WITH EACH TISSUE

Development of scrapie in goats inoculated intracerebrally with various tissues from a scrapie-affected goat.

From Figure 3 it will be seen that suspensions of frontal cortex, brain stem, pituitary and adrenal gland produced the disease in all animals inoculated, and that the disease appeared at approximately the same time after inoculation (between seven and 12 months) in all animals. The spleen suspension produced scrapie in all of three goats, but in this instance the incubation period was from 12 to 23 months. To the time of writing, 24 months after inoculation, the pancreas and liver suspensions have produced the disease in two of three, and one of three animals respectively, with an incubation period of 12 months or longer, and the disease has not occurred in any of the three animals inoculated with a suspension of thyroid gland. If one postulates that a reduction in the scrapie-producing potential of an inoculum is accompanied by a lengthening of incubation period and a reduction in take among inoculated animals, then these results suggest that the scrapie-producing potential of suspensions of frontal cortex, brain stem, pituitary and adrenal was approximately the same, and that the potential of spleen, pancreas, liver, and thyroid was progressively less.

Scrapie occurred 23 months after inoculation in a goat injected intracerebrally with 1·0 ml. of a 10 per cent suspension of frontal cortex from a clinically healthy goat, this being the only case of scrapie to the time of writing, that has appeared in the group of animals inoculated with suspensions of various tissues obtained from this animal. This case of scrapie was clinically characteristic and histological examination of the brain confirmed the clinical diagnosis. Observation of the remaining animals in this group continues. The appearance of this case of scrapie following intracerebral inoculation of brain suspension from a clinically normal goat makes it necessary to elaborate briefly on the circumstances of this unexpected occurrence. For this reason the history of the clinically normal goat from which tissues were obtained, and the husbandry of the animal that became affected with the disease are summarised as follows

History of the clinically healthy goat. A female born at Compton 9.2.52 and kept in a non-scrapie environment. Injected subcutaneously on 30.10.56 with 40 ml. of sheep blood, obtained from Moredun Institute containing tick-borne fever rickettsiae. Did not develop tick-borne fever. On 20.5.57, being then clinically normal, slaughtered to provide normal tissues for inoculation. These inoculations were carried out two days before the inoculations with tissues from the scrapie-affected goat.

The following is the origin of the tick-borne fever blood. Three lambs were inoculated at Moredun Institute with tick-borne fever blood in April 1956; two were out of ewes naturally affected with scrapie. During the period April/June 1956 blood from these three lambs was sub-inoculated into lambs with no history of scrapie. In October 1956 a pool of blood from these latter lambs was sent to Compton for inoculation of goats. Three goats were inoculated, one of which provided the tissues for the inoculations described above when slaughtered seven months later; the other two remain alive and have shown no evidence of disease.

Husbandry of the animal inoculated intracerebrally. A castrated male born at Compton February 1957 in a non-scrapie environment out of clinically normal dam by a clinically normal sire. It was inoculated at 12 weeks of age and maintained in a non-scrapie environment. It was removed from the non-scrapie environment on 20.2.59 to a loose-box in a building in which a number of animals affected with scrapie were housed, but was not in direct contact with them. It developed scrapie one month later and was slaughtered on 1.5.59.

Attempts to Spread Scrapie by direct Contact between Goats clinically Affected with Scrapie and Non-inoculated Goats.

Table 1 gives details of 28 animals, the progeny either of non-scrapie parents or of scrapie-affected dams by a non-scrapie sire, that have been kept in a scrapie environment for 24 to 34 months, to the time of writing. None has shown evidence of scrapie. Observation of all is being continued.

TABLE 1

DETAILS OF 28 GOATS MAINTAINED IN A SCRAPIE ENVIRONMENT WITHOUT DEVELOPING THE DISEASE

Group	*Number and sex*	*Age when put in scrapie environment*	*Length of time* in scrapie environment : months*
1. Progeny of normal parents	1 entire male	9 months : served scrapie-affected females	30
2. Progeny of normal parents	16 castrated males	3 months	24
3. Progeny of scrapie-inoculated dams by a non-inoculated sire	2 castrated males 2 females	From birth	34
4. Progeny of scrapie-inoculated dams by a non-inoculated sire	7 castrated males	From birth	26

*to the time of writing.

DISCUSSION

The observations reported in this paper have confirmed our previous finding that scrapie will occur in a high proportion of goats, possibly 100 per cent., inoculated intracerebrally with 1·0 ml. of the supernatant from a lightly centrifuged 10 per cent suspension in physiological saline of brain from a scrapie-affected goat. They have shown, further, that intracerebral inoculation of goats with saline suspensions of certain other tissues, e.g. pituitary, adrenal, spleen, pancreas and liver from a scrapie-affected goat will also produce the disease, but they indicate that any individual inoculum prepared from the tissues of a scrapie-affected animal has its own individual scrapie-producing potential. In some cases this potential is great enough to produce the disease in all goats inoculated after a minimum incubation period, but in others a lowering of the potential is reflected in a lengthening incubation period and a reduced take among the animals inoculated. This general principle is demonstrated by the results given in Figure 3. It would appear, also, that if a route of inoculation other than the intracerebral is used, the scrapie-producing potential may be altered (Fig. 1). Again, evidence is presented to indicate that brain suspension from a scrapie sheep inoculated into goats is less certain to produce the disease, and will take longer to produce it, than if scrapie goat brain is injected into goats.

From these various observations it is concluded that the optimum conditions for producing scrapie in goats are provided by intracerebral inoculation of a suspension of scrapie goat brain. If the character of the inoculum is varied, or if another route of inoculation is used, then variations in response, shown by lengthening of the incubation period and reduction in take among inoculated animals, may be expected. From this conclusion it follows that experiments in which the scrapie-potential of the inocula may have been reduced, e.g. those designed to study the resistance of the scrapie agent to physical or chemical agents, must not be discontinued until long after the time at which clinical evidence of the disease might, under normal circumstances, have been expected. Indeed, in spite of obvious practical difficulties, it might be argued that those experiments that depend for their interpretation on the appearance of scrapie among the animals inoculated ought not to be discontinued until the disease has in fact developed, or until the animals have reached the end of their natural lives.

There has been no indication in these further experiments on scrapie in goats that the age (within the range of a few days to six months), sex, or breeding of the inoculated animals has an effect on the eventual appearance of the disease. They confirm, however, that there is wide variation among individual animals in the clinical manifestations of the disease, and it has been noted that a sudden onset of clinical abnormality is less usual than a slowly progressing deviation from normality that results after a few weeks in the characteristic syndrome previously described.

The occurrence of scrapie in one goat 23 months after it had been inoculated intracerebrally with a suspension of brain from a clinically healthy goat was an unexpected incident. It would appear to be unprofitable at the moment to speculate on this observation, but further experiments are in progress to examine its meaning.

CONCLUSIONS

A fifth intracerebral passage of scrapie in six goats has, together with earlier passages previously reported, produced the disease in all of 56 animals inoculated.

Comparison of the intracerebral and subcutaneous routes of inoculating scrapie brain suspension into groups of ten goats showed a significantly longer incubation period in the animals injected subcutaneously.

Multiple subcutaneous inoculations of scrapie brain suspension, up to a maximum of 10 ml. on 50 consecutive days, produced scrapie in all of four goats after an incubation period that was no shorter than that observed when a single 1·0 ml. dose is inoculated intracerebrally, but significantly shorter than when a single subcutaneous injection is given.

Treatment of scrapie goat brain suspension with ribonuclease or deoxyribonuclease did not reduce its ability to produce scrapie when inoculated intracerebrally into goats.

Scrapie sheep brain sent from Moredun Institute to Canada and back to Compton over a period of 42 days was still capable of producing scrapie when inoculated intracerebrally into sheep and goats.

No abnormal rectal temperature was recorded in either of two goats throughout the incubative and clinical stages of scrapie.

Brain suspensions prepared from each of five Swaledale sheep naturally affected with scrapie produced the disease in seven of 25 Cheviot sheep and in two of ten goats inoculated intracerebrally, 30 months after inoculation to the time of writing, but the incubation period in both species was longer than is usually observed.

Confirmation was obtained of the previous observation that cerebrospinal fluid from a scrapie-affected goat is capable of producing scrapie when inoculated intracerebrally into goats.

Suspensions of pituitary gland, adrenal gland, spleen, pancreas, and liver from a scrapie-affected goat produced scrapie when inoculated intracerebrally into goats, but the incubation period and the take within a 24-month period of observation varied according to the suspension used; a similar suspension of thyroid gland failed to produce the disease over the same period after inoculation.

A normal goat inoculated intracerebrally with 1·0 ml. of a suspension of brain from a clinically normal goat, the history of which is given, developed scrapie 23 months later. Further experiments are in progress to examine the significance of this unexpected occurrence.

Twenty-eight non-inoculated goats housed in a scrapie environment over periods varying from 24 to 34 months have, to the time of writing, shown no evidence of disease.

ACKNOWLEDGMENTS

We wish to express to Dr. W. S. Gordon, C.B.E., and to Dr. H. H. Holman our thanks for their keen interest in this work, advice and encouragement; and to Mr. J. Elliot, Manager of the Isolation Compound, our appreciation of the care and trouble taken over the housing and management of the animals.

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BIOCHEMICAL AND HAEMATOLOGICAL OBSERVATIONS ON THE BLOOD AND CEREBROSPINAL FLUID OF CLINICALLY HEALTHY AND SCRAPIE-AFFECTED GOATS

By

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INTRODUCTION

During the course of experiments described by Pattison and Millson (1960) on scrapie produced experimentally in goats, the opportunity arose to make certain biochemical and haematological measurements on clinically healthy and scrapie-affected animals. This paper records the results of these observations.

MATERIALS AND METHODS

Blood was examined from ten clinically healthy and ten scrapie-affected goats in advanced stages of the disease. There were eight castrated males and two females in each group. The eight castrated healthy animals had previously been inoculated intracerebrally with various tissues from a normal goat (Table 1); the two healthy females were picked at random from the Compton herd of crossbred animals. Cerebrospinal fluid (C.S.F.) from three healthy and five scrapie-affected goats was examined. Further particulars of the sex and age of the animals and the inocula used are given in Table 1.

Haematology. The animals were bled from the jugular vein into Paul and Heller's mixture of potassium and ammonium oxalate as anticoagulant. Blood specimens were examined on three consecutive days. The results in Table 2 are the average of these three measurements.

The erythrocyte, leucocyte, and differential counts were carried out as described by Holman (1950). The haemoglobin was measured as alkaline haematin in a Lovibond comparator; the packed cell volume was recorded after spinning for five minutes in a Hawksley microhaematocrit centrifuge; specific gravity of whole blood and plasma was measured by using different proportions of chloroform and benzene at a standard temperature of 20°C.; the viscosity was measured at 20°C. in a Hess viscometer.

Biochemistry of blood. The blood was collected in heparinised bottles and sodium fluoride was added to a separate sample intended for blood sugar determination. One sample from each goat was examined.

The following examinations were made:

(a) Total protein, albumin and globulin by the method of Gornall, Bardawill and David (1949); readings were made in a Unicam spectrophotometer S.P. 500 using 1 c.m. curvettes at a wavelength of 540 $m\mu$.

(b) True sugar, by the method of King and Garner (1947).

(c) Urea, by the method of Archer and Robb (1925).

(d) Calcium, by the oxalate-permanganate method of Clark and Collip (1925).

Group	*No. of goat*	*Inoculated i.c. with 1·0 ml. of a suspension of:*	*Sex**	*Age when sampled : months*	*Time between inoculation and sampling : months*	*Duration of disease at time of sampling : months*
Clinically healthy Blood taken	1	Normal spleen	Male	21	18	—
	2	,, pancreas	,,	20	17	—
	3	,, ,,	,,	22	19	—
	4	,, brain	,,	21	18	—
	5	,, ,,	,,	22	19	—
	6	,, liver	,,	20	17	—
	7	,, pituitary	,,	22	19	—
	8	,, thyroid	,,	20	17	—
	9	Not inoculated	Female	20	—	—
	10	,, ,,	,,	20	—	—
Clinically healthy, C.S.F. taken	11	Normal adrenal	Male	13	10	—
	12	Not inoculated	Female	24	—	—
	13	,, ,,	,,	84	—	—
Scrapie-affected Blood taken	14	Scrapie brain	Male	20	17	2
	15	,, ,,	,,	20	17	3
	16	,, spleen	,,	21	18	2
	17	,, ,,	,,	21	18	4
	18	,, liver	,,	21	18	2
	19	,, pancreas	,,	20	17	1½
	20	,, C.S.F.	,,	18	14	½
	21	,, ,,	,,	18	14	2
	22	,, brain**	Female	32	27	1½
	23	,, ,,	,,	32	27	2
Scrapie-affected C.S.F. taken	24	Scrapie brain	Male	13	9	1
	25	,, ,,	,,	13	12	2
	26	,, ,, **	Female	18	13	½
	27	,, ,,	,,	21	16	1½
	28	,, ,,	,,	21	16	2½

*All males were castrated at about six weeks of age.
**5·0 ml. inoculated subcutaneously

TABLE 2

RESULTS OF BIOCHEMICAL AND HAEMATOLOGICAL EXAMINATIONS

Blood of ten clinically healthy and ten scrapie-affected goats

Biochemical results: plasma constituent	*Range for the normal goats*	*Number of scrapie-affected goats within normal range*	*Values for scrapie-affected goats outside normal range*
Total protein — g./100 ml.	6·70—7·85	4	6·60, 7·95, 8·45, 8·75 8·95, 9·00
Albumin — g./100 ml.	3·25—4·90	9	3·10
Globulin — g./100 ml.	2·30—4·60	6	4·70, 4·75, 4·80, 5·05
True sugar* — mg./100 ml.	56—82	6	84, 91, 122, 160
Cholesterol — mg./100 ml.	81—176	9	296
Urea — mg./100 ml.	30—39	6	40, 40, 41, 44
Chloride — mg./100 ml.	380—415	9	344
Calcium — mg./100 ml.	10·9—12·9	8	10·6, 10·7
Phosphorus — mg./100 ml.	1·5—5·0	9	5·5
Haematological results			
Haemoglobin — g./100 ml.	9·5—13·6	10	—
Packed cell volume — %	26—39	10	—
Erythrocytes — 10^6/c.mm	12—18	7	19, 19, 20
Specific gravity: Aq. = 1000			
Plasma	1023—1029	10	—
Whole blood	1045—1056	9	1060
Viscosity of whole blood — Aq. = 1	3·6—5·8	9	6·0
Leucocytes — 10^3/c.mm	9—15	5	7, 17, 18, 19, 24
Differential count:			
Neutrophils — %	21—44	5	50, 57, 58, 62, 68
Lymphocytes — %	53—79	5	31, 38, 39, 47, 47
Monocytes — %	0—2	8	3, 3
Eosinophils — %	0—4	9	5
Basophils — %	0—1	10	—

*Measured on whole blood.

(e) Phosphorus, by the method of Fiske and Subbarow (1925).
(f) Cholesterol, by the alcohol-ether extraction method of Sackett (1925).
(g) Chloride, by the method of Schales and Schales (1941).

The protein, true sugar, chloride, calcium, and phosphorus tests were carried out on the day of collection; the urea and cholesterol tests were performed after storage of the plasma overnight at 4°C. The measurement of true sugar was carried out on whole blood; all other estimations were carried out on plasma.

Biochemistry of cerebro-spinal fluid. The C.S.F. was removed by puncture through the cisterna magna while the animal was under nembutal anaesthesia. One sample from each animal was examined. The specimens were stored at -10°C. for approximately three months, when total protein, chloride, sugar and urea were estimated. Total protein was measured by visual matching using a Grey's proteinometer. Chlorides, sugar and urea were measured by the methods described above.

RESULTS

The haematological and blood biochemical measurements made are given in Table 2, and the C.S.F. measurements in Table 3. From these tables it will be seen that no constant difference was detected between the normal and the scrapie-affected goats. As only a limited number of biochemical tests was used in this work, it is possible that a more detailed study might reveal some characteristic deviation from the normal in scrapie-affected animals.

TABLE 3

RESULTS OF BIOCHEMICAL EXAMINATION OF THE C.S.F.

Three clinically healthy and five scrapie-affected goats

Constituent: mg./100 ml.	*Clinically healthy goats*	*Scrapie-affected goats*
Total protein	20 : 20 : 43	10 : 15 : 15 : 23 : 45
Glucose	52 : 53 : 54	33 : 43 : 49 : 50 : 65
Chloride	451 : 454 : 462	433 : 440 : 447 : 451 : 490
Urea	19 : 30 : 43	11 : 20 : 22 : 24 : 27

CONCLUSIONS

The haematological and biochemical tests used failed to demonstrate any significant difference between the blood of ten clinically healthy and ten scrapie-affected goats of comparable age and sex. Similarly, the biochemical tests used showed no significant difference between the cerebro-spinal fluid of three clincally healthy goats and five scrapie-affected goats.

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We wish to thank Mr. I. H. Pattison for obtaining the samples of blood and C.S.F. and for advice and encouragement throughout this work, Dr. H. H. Holman for his help with the haematology, and Mr. R. J. Garner for generously extending facilities for the biochemical investigations.

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CLINICAL BIOCHEMISTRY OF FARM ANIMALS

II. AMINO-ACID AND PROTEIN PATTERNS OF THE BODY FLUIDS OF CATTLE AND SHEEP IN DISEASE

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INTRODUCTION

Biochemical changes, reflected in an abnormal composition of body fluids, sometimes accompany and often precede other manifestations of disease. They are important in understanding the fundamental processes involved and may be helpful as aids in diagnosis.

Convenient methods have recently become available to investigate amino-acid patterns and protein components of blood and urine. When applied to cases of human hepatic disorders and especially the amino-acidurias, characteristic patterns divergent from the normal are observed (Walshe, 1953; Dent and Walshe, 1954; Smith, 1958). Severe liver damage in man (Martin and Davies, 1955; Owen and Robertson, 1956) and farm animals (Campbell, 1957; Rooney, 1957) causes a rise in the γ-globulin. It is also well known that bacterial and viral infections are accompanied by alterations in the various globulin components of plasma. Mould and Stephen (1959) have used column electrophoresis for the fractionation of the γ-globulins in certain conditions affecting cattle. It is desirable to extend the use of these techniques to a variety of farm animal ailments and some preliminary results of such studies in plant poisoning and other pathological conditions are now reported.

MATERIALS AND METHODS

Paper chromatography and paper electrophoresis. Applications of these techniques to body fluids have already been described (Coulson, Davies, and Evans, 1959).

Ragwort supply. Senecio jacobaea plants in flower were collected in September, 1957, oven-dried at 60°C and coarse milled. Fresh supplies were gathered as required for direct feeding, in June, 1958.

Extraction and chromatography of Senecio *alkaloids.* Milled ragwort (3g.) was exhaustively extracted with ethanol (120 ml.). The residue after evaporation of the ethanol was triturated with 100 ml. of 2 per cent HCl and filtered. The filtrate was cleared of pigments by ether and chloroform extraction. On making alkaline with ammonia, the bases were liberated and extracted into chloroform, which was finally evaporated to a small bulk. For chromatographic separation of the alkaloid components, the extract (0·2 ml. $CHCl_3$ extract, equivalent to 1 g. ragwort) was spotted on to strips (5 cm. ×45 cm.) of Whatman No. 4 paper. Ascending chromatography was used, employing the following solvents: (1) *n*-butanol/acetic acid/water 100/30/just saturated; (2) same solvents, but 100/10/just saturated; (3) same solvents but 100/4/just saturated; (4) *n*-butanol/formic acid/water, 100/10/just saturated; (5) 75 per cent iso-propanol, 2N with respect to HCl. The alkaloid areas were located by using Dragendorff's reagent as a dip (5 per cent potassium bismuth iodide in 0·5 per cent HCl) when they appeared as reddish brown spots on a yellow background.

Bracken supply. Pteridium aquilinum was collected during June and July and dried at low temperature (30 to 40°C).

Fibrinogen estimations. Method of King and Wootton (1956).

Plasma "amino-acid" nitrogen. Method of Russell (1944).

Liver function tests. (1) Thymol turbidity test as given by Maclagan (1944, 1947), and Huerga and Popper (1949). Protein turbidity tubes* were used as comparison. (2) Iodine-Potassium iodide test as given by Belle and Chini (1954) and quoted by Lloyd (1957).

Haematological methods. Those previously described by Evans, Evans and Hughes, (1954).

Field cases. These were made available through the Veterinary Investigation Centre, Bangor; details are given in the text.

RESULTS

Ragwort Experiments

The ragwort, *Senecio jacobaea,* used to produce experimental poisoning, contained two chromatographically separable alkaloids (Solvents 1 to 4). A crystalline alkaloid (jacodine alias jacobine) isolated from the marsh ragwort, *S. aquaticus* (Evans and Evans, 1949) had the same RF as the faster moving component and this is probably jacodine (or jacobine), the other being senecionine. Estimation was possible by comparing the intensities of these spots.

RF of Spot 1: (1) 0·88, (2) 0·57, (3) 0·54, (4) 0·51.
RF of Spot 2: (1) 0·80, (2) 0·73, (3) 0·66, (4) 0·65.
RF of jacodine: (1) 0·80, (2) 0·73, (3) 0·66, (4) 0·65.

Ragwort poisoning experimentally induced. A three month old Ayrshire cross heifer calf was placed on a ragwort diet on 15th May, 1958. Feeding was started at 0·5 lb. of ragwort per day mixed with dried grass. After 14 days this was increased to 1 lb. per day for 22 days and at this stage about 25 lb. of dry ragwort had been consumed. Because of difficulties in persuading the animal to eat the dried material, the animal was then fed fresh ragwort for 18 days. A further 31 lb. (dry matter) were consumed, to give a total of 56 lb. dry ragwort. Blood and urine samples were taken regularly over this period. At this stage there were no outward signs of poisoning and the animal was turned out to grass, but kept under observation. During the first few days grazing was normal; afterwards, the animal became progressively more prone to lie down and it was obvious that it was not well. It was brought indoors after nearly 14 days in the field and died on the same day (18/7/58). Post-mortem showed extensive oedema, especially of the intestinal tract, and the liver presented signs of seneciosis, confirmed histologically. A blood sample was taken for serum only two hours before death. Table 1 gives the haematological data together with the results of two liver function tests.

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*Messrs Gallenkamp Ltd.

TABLE 1

HAEMATOLOGICAL FINDINGS IN RAGWORT POISONING

Date	*Red cell count* ($\times 10^6$/*cmm.*)	*White cell count* ($\times 10^3$/*cmm.*)	*Platelets* ($\times 10^3$/*cmm.*)	*Differential leucocyte count* (*relative* %)				
				L	*N*	*M*	*E*	*B*
Pre-expt.	8·5	10·2	780					
20.5.58	8·5	13·2	840	42·0	54·0	1·5	2·0	0·5
29.5.58	8·5	8·7	854	44·5	48·0	3·0	3·5	1·0
5.6.58	8·6	8·2	790	51·5	38·0	3·5	6·5	0·5
12.6.58	8·4	8·5	660	53·0	35·5	6·0	5·5	—
*19.6.58	8·0	7·9	550	53·5	30·5	5·5	10·0	0·5
26.6.58	8·0	7·1	490	62·0	23·5	2·5	11·5	0·5
5.7.58	8·3	7·4	480	58·5	14·5	5·0	20·5	1·5
18.7.58	—	—	—					

L=lymphocytes *N*=neutrophils *M*=monocytes *E*=eosinophils *B*=basophils

*Iodine test became positive here

Thymol turbidity test negative throughout

The fall in platelets was accompanied by a fall in total white cells, a sharp fall in neutrophils, a slow rise in lymphocytes and a rapid rise in eosiniphils. The red cells did not change perceptibly and there was no change in the serum proteins up to the beginning of July (Table 2). No samples were taken from 5.7.58 to 18.7.58 during which time the animal was out on grass. In the last sample taken two hours before death the γ-globulin was found to be elevated by some 50 per cent. (Fig. 3). There was no abnormality in the plasma free amino-acid chromatograms (Plate I) until the last sample investigated which showed clearly a very large increase in the total concentration of free amino-acids. It also showed spots not detectable in normal plasma at the concentration used. They have been identified tentatively as taurine, β-amino-*iso*-butyric acid and possibly ornithine and ethanolamine. No sample of urine was obtained at the time the blood sample was taken, but the previous sample (5.7.58) was normal.

TABLE 2

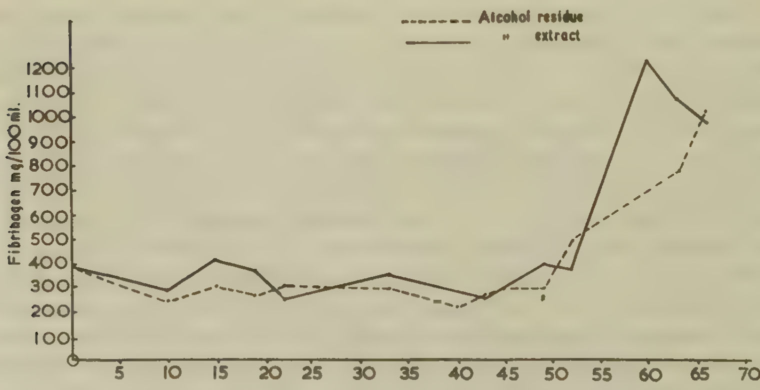
SERUM PROTEINS (RELATIVE %) AND AMOUNT OF RAGWORT CONSUMED

Date	*Albumin*	*Globulins*			*Amount eaten approx. (D.M. in lb.)*
		α	β	γ	
15.5.58	43	22	10	25	0
20.5.58	37	26	12	25	2½
29.5.58	45	22	10	23	8
5.6.58	44	22	12	22	13
12.6.58	42	22	15	21	20
†19.6.58	43	22	11	25	25
26.6.58	48	20	10	22	35
5.7.58	45	20	11	24	56* stop
(turned out to grass)					←
18.7.58	38	18	11	33	56

*This probably corresponds to an estimate of between 1 and 2 g. of the total alkaloids.
†Iodine test became positive here.

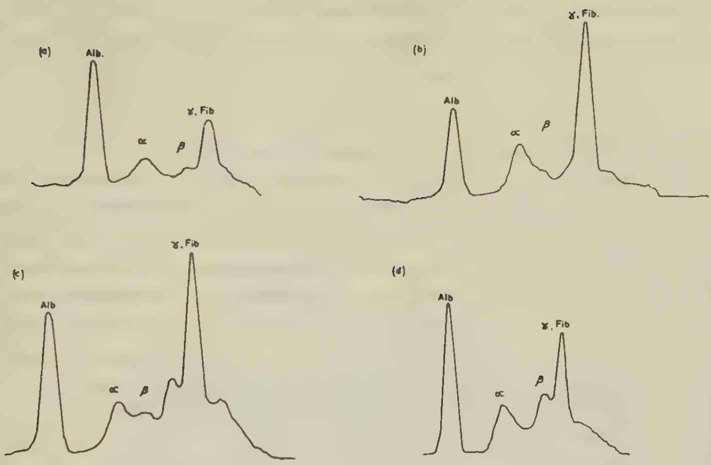
Field case. Plasma from a calf suffering from clinical ragwort poisoning, confirmed by macroscopic and microscopic examination of the liver, was examined by electrophoresis. Serum prepared from this plasma by the addition of calcium ions was examined by the

Fig. I.



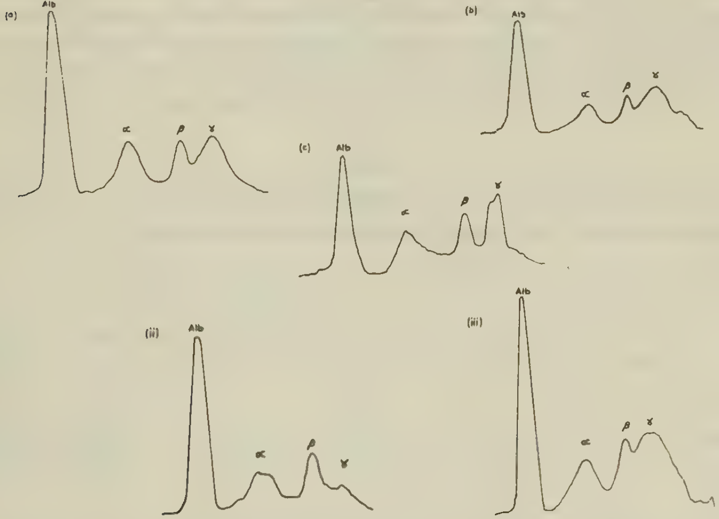
Bracken poisoning. Fibrinogen content of bovine plasma. (a) Alcohol residue diet. (b) Alcohol extract diet. Fibrinogen (mg./100 l.) versus days of experiment.

FIG. 2.



Colour density curves of calf plasma proteins (separated on "Oxoid" strips, dyed with Azocarmine B), (a) Normal, (b) experimental bracken poisoning, (c) field case of bracken poisoning produced by bracken rhizomes (Red bullock: white cells $1{\cdot}9 \times 10^3$/cmm.) Animal died next day. In cases (b) and (c) note increase in the fibrinogen component. The serum proteins were normal. (d) Ragwort poisoning (field case).

FIG 3



(i) Experimental ragwort poisoning (started 15.5.58). Colour density curves of calf serum proteins (separated as in Fig. 2). (a) Normal (5.7.58), (b) abnormal two hours before death (18.7.58), (c) field case (serum prepared from plasma). (ii) Copper deficiency (field case). Colour density curve of calf serum proteins (separated as in Fig. 2). (iii) Lead poisoning (field case). Colour density curves of fractionated serum proteins (separated as in Fig. 2).

same method. The fibrinogen content of the fractionated plasma proteins appeared to be higher than normal (Fig. 2d.), but as the serum, prepared from the plasma, had a higher γ-globulin content, (Fig. 3c.) the fibrinogen may not have been outside the normal range.

Bracken Experiments

Bracken poisoning experimentally induced. A hot water concentrate of fresh bracken (4 kg.) prepared according to the directions of Evans, Evans, Chamberlain and Thomas (1959) was given daily by drench to a six months old Ayrshire/Hereford cross heifer fed on an otherwise normal diet. Clinical bracken poisoning was produced in three weeks. Periodic blood samples were taken for investigation. The serum proteins of this animal, suffering from clinical bracken poisoning, were within normal limits even in the acute stages of the disease (Table 3). Accompanying the sharp fall in leucocytes and platelets, was a noteworthy rise in the plasma fibrinogen (Fig. 1). This was a constant finding in the later stages of this condition. The plasma fibrinogen levels were definitely abnormal and rose to as much as 1300 mg./100 ml. plasma (normal range: 200 to 400 mg./100 ml. plasma by this method).

Field cases. Bracken poisoning caused by rhizomes does not appear to have been recorded before, but there is little doubt that in late April, 1958, an outbreak in the Pwllheli area was caused by them. Two yearling, red and white shorthorn bullocks developed characteristic bracken poisoning of the enteric type after eating rhizomes which had been exposed by ploughing during March and April, 1958; both animals eventually succumbed. Plasma and serum samples were obtained at the critical stage of the disease.

TABLE 3

SERUM PROTEINS (RELATIVE %) AND BLOOD CELL COUNTS IN BRACKEN POISONING

Date	*Albumin*	*Globulins*			*Blood Cells*		
		α	β	γ	*Red* (× 10^6/*cm.*)	*White* (× 10^3/*cmm.*)	*Platelets* (× 10^3/*cmm.*)
29.5.58	36	24	11	29	8·5	13·2	640
5.6.58	46	18	13	23	8·4	11·8	660
12.6.58	40	25	16	19	8·4	7·4	380
19.6.58	36	25	14	25	8·1	2·6	5
Average	39·3 ± 4·7	23 ± 3·4	13·5 ± 2·1	24 ± 4·2			

± standard deviation.

The serum proteins appeared to be within the normal range, but both animals had raised plasma fibrinogen levels. (Fig. 2d).

Other Pathological Conditions

Copper deficiency

Cattle. Serum samples from five calves with biochemically established copper deficiency were used for paper electrophoresis and for amino-acid chromatography. Figures for serum protein values (Table 4) showed that the main feature was a decreased γ-globulin and an increase in the β-globulin fraction (Fig. 3). The free amino-acid patterns of the serum samples of two of the animals did not show a divergence from normal. One of the animals had an enlarged liver on post-mortem examination.

Sheep. Blood samples were obtained from fifteen ewes on three separate farms where copper deficiency was established. All had lambed and all lambs were affected by swayback. Blood copper figures were supplied by the Biochemistry Department, Veterinary Laboratories, Weybridge. A blood sample from a lamb suffering from this disorder showed 0·01 mg. Cu/100 ml. The lamb which was given one teaspoonful of a 1 per cent $CuSO_4$ solution daily for about a fortnight survived. A blood sample was taken at the end of this period. The plasma free amino-acid patterns of the fifteen ewes did not differ from those of normal ewes after lambing. The blood copper values were from 0·008 to 0·02 mg./100 ml. blood (normal values; 0·07 to 0·1 mg./100 ml. blood). The pattern of plasma free amino-acids in the one lamb suffering from swayback did not appear abnormal and showed no change after recovery.

TABLE 4

COMPOSITION OF VARIOUS SHEEP AND CATTLE SERA (RELATIVE %)

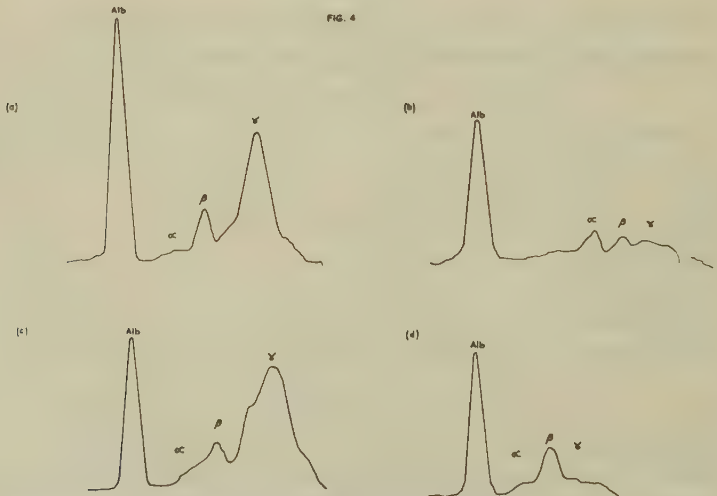
Condition	*No.*	*Albumin*	*Globulin*			*Ref.*
			α	β	γ	
Calf						
Normal	3	44·3 ± 1·5	21·3 ± 1·2	11·3 ± 1·4	23 ± 1·7	*
Bracken poisoning (see sep. table)						
Ragwort poisoning (see sep. table)						
Copper deficiency	5	44·4 ± 4·3	25·6 ± 3·1	18·8 ± 2·6	11·2 ± 3·5	
Lead poisoning	2	31 ± 3	18 ± 0	15 ± 1·5	35·5 ± 1·5	
Sheep						
Normal	5	44·2 ± 2·9	4·2 ± 1·8	13·2 ± 3·0	38·2 ± 5	*
Hypocalcaemia	1	46	10	13	31	
Hypophosphorosis	1	25	6	13	56	
Coccidiosis	1	49	8	24	19	

*Coulson, Davies, and Evans, (1959).

Kale poisoning. Two milch cows were found to be suffering from kale poisoning caused by uncontrolled access to a field of marrow-stem kale. Urine and plasma samples were examined for free amino-acids by paper chromatography. The most severe case had a dramatic proteinuria similar in character to acute human nephritis. Hydrolysates of the proteins precipitated from the urine were also examined for amino-acids by paper chromatography. One animal (1) showed a severe haemoglobinuria allied with severe anaemia; the other (2) had less pronounced symptoms. The haemoglobin ratio was: $1/2 = 23{\cdot}5/41{\cdot}5$. The red cell counts were: (1), $2{\cdot}2 \times 10^6$/cmm., (2), $5{\cdot}3 \times 10^6$/cmm. Amino-acid chromatograms of the plasma samples showed no abnormality. The amino-acid chromatograms of the protein-freed urine hydrolysates were quite normal. The hydrolysed protein isolated from the urine gave a qualitatively normal protein amino-acid pattern where some protein oxidation has occurred; cysteic acid and methionine sulphone were present (Davies, 1958).

Lead poisoning. Serum samples were obtained from two six-month old calves with suspected lead poisoning. The clinical diagnosis was based on access to flaking lead paint. They were injected with the calcium disodium salt of ethylenediamine tetra-acetic acid two days before the blood samples were taken. The serum proteins were examined by paper electrophoresis and the free amino-acids of serum by paper chromatography. Serum amino-acid patterns were normal. The electrophoretic separation of the serum proteins showed that they diverged from the normal with a large increase in γ-globulin at the expense of the other protein fractions (Table 4; Fig. 3 iii).

FIG. 4



Colour density curves of sheep serum (separated as in Fig. 2). Field cases. (a) Normal, (b) hypocalcaemia, (c) hypophosphorosis, (d) coccidiosis.

Hypocalcaemia. This condition was encountered in a ewe a few days before parturition. Two days before lambing the ewe was injected with 50 ml. of a 20 per cent solution of calcium borogluconate. The animal died soon after lambing. A blood sample was taken one day before it lambed. Determinations of blood calcium, magnesium and acetone were carried out at the Biochemistry Department, Veterinary Laboratories, Weybridge. Serum was used for electrophoresis. The figure of 4·0 mg./100 ml. for blood calcium of the single case examined was low, whilst the acetone and magnesium values were within normal limits. The free amino-acids of the plasma appeared to be identical with those of normal sheep, but the γ-globulin of the serum was twice the normal value (Table 4; Fig. 4b).

Hypophosphorosis. The one case of this condition in a ewe appeared to have normal free plasma amino-acids. The serum protein values, however, were divergent from normal (Table 4; Fig. 4c). The albumin was almost half the normal value whilst the γ-globulin was about 50 per cent higher.

Coccidiosis. A serum sample obtained from a lamb with established coccidiosis was examined by paper electrophoresis. It had an increased β-globulin level (Table 4; Fig. 4d).

DISCUSSION

In ragwort poisoning, gross changes in the plasma amino-acid pattern and a rise in the γ-globulin only occurred shortly before death. The ability of the iodine-potassium iodide test to detect liver damage earlier than the other techniques used supports the claims made by Belle and Chini (1954) and Lloyd (1957) that it is valuable for early diagnosis of this condition. The formation of the precipitate with iodine appears to be unconnected with the change in the γ-globulin fraction since the test becomes positive well before there is any increase in γ-globulin.

Platelets fell progressively but in the early stages they were not below normal limits—unfortunately no figures are available for the terminal phase. Harris, Henderson and Chen (1957) have reported slight hypothrombinaemia after the administration of *S. longilobus* to sheep. The differential leucocyte counts show a marked eosinophilia and neutropenia: it will be interesting to see whether this is a constant finding in ragwort poisoning.

The normal pattern of plasma-free amino-acids of animals poisoned by bracken shows that the liver is not seriously impaired. The normal serum protein levels found in these cases support this conclusion. Evans *et al.* (1954) and Heath and Wood (1958) also found that the plasma proteins, other than fibrinogen, were not altered. The cause of the fibrinogen increase deserves further attention.

In other diseases we merely wish to record our findings in the few field cases made available to us. It is hoped in due course to examine a larger series. In lead poisoning the steep rise in γ-globulin in the two calves examined is presumably a result of liver damage. Kale poisoning, brought about a distinct anaemia in one of the two milking cows and this resulted from a lowered erythrocyte level, but no hepatic insufficiency was noted as claimed by Cohrs, (1943) since the plasma free amino-acid patterns were normal in both cases. Although Evans (1951) appears to be the first to have drawn attention to kale poisoning in this country, Rosenberg (1943) initially reported "kale anaemia" in cattle. The marked decrease in γ-globulin and increase in β-globulin in four out of five of the copper deficient calves is contrary to the finding of Campbell (1957).

In the one case of coccidiosis examined in sheep there was a raised β-globulin and a lowered γ-globulin value. Although β-globulin is often increased in a chronic condition and coccidiosis is usually a chronic condition, it is known to behave, on the whole, uncharacteristically (Antweiler, 1957). The ewe affected with severe hypophophorosis, had an increased γ-globulin fraction which could be due to the animal suffering from an accompanying infection.

CONCLUSIONS

Observations on plasma and urine amino-acids and serum proteins in cattle and sheep affected with various pathological conditions, are reported.

Ragwort poisoning induced experimentally in a calf only produced gross changes in the plasma amino-acid patterns and serum proteins shortly before death. The iodine test of Belle and Chini was a more useful diagnostic aid. An eosinophilia and a neutropenia were noted.

In cattle, field cases of bracken poisoning and the experimentally induced disease produced no gross change in plasma or urinary amino-acid patterns nor in the serum protein patterns, but plasma fibrinogen was markedly increased.

A limited number of other bovine and sheep disorders have been explored by means of paper chromatography and paper electrophoresis. A more extensive series is needed before firm conclusions can be drawn.

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LEGENDS

Plate 1. (a) Normal bovine plasma free amino-acid pattern (two dimensional paper chromatography; ninhydrin location). Solvents: 1. phenol-water/ammonia (80:20/3% atmosphere). ii. *n* Butanol-acetic acid-water (62:12:26). 1. Glutamic acid. 2. Serine. 3. Glycine. 4. Threonine. 5. Alanine. 6. Citrulline. 7. Glutamine. 8. Histidine. 9. Lysine. 10. Arginine. 11. Proline. 12. Tyrosine. 13. α-amino-*n*-butyric acid. 14. Valine+methionine. 15. Phenylalanine. 16. Leucine+*iso* leucine. 17. Aspartic acid. 18. Unknown. 19. Asparagine. 20. Cyst(e)ine. 21. Unknown.

Plate 1. (b) Experimental ragwort poisoning (18.7.58). Plasma amino-acids two hours before death (see heavier pattern). 1. Glutamic acid. 2. Serine. 3. Glycine. 4. Threonine. 5. Arginine. 6. Citrulline. 7. Glutamine. 8. Histidine. 9. Ornithine. 10. Arginine. 11. Proline. 12. Tyrosine. 13. α-Amino-*n*-butyric acid. 14. Valine+methionine. 15. Phenylalanine. 16. Leucine+*iso*-leucine. 17. Aspartic acid. 18. 3,4-Dihydroxy-phenylalanine. (?) 19. Asparagine. 20. Unknown. 21. Unknown. 22. Lysine. 23. β-amino-*iso*-butyric acid. 24. Methionine sulphoxide. 25. Unknown. 26. Ethanolamine(?). 27. γ-amino-*n*-butyric acid. 28. Cyst(e)ine. 29. Taurine.

Plate 1. (c) Experimental ragwort poisoning (5.7.58). Amino-acids of urine hydrolysate (normal pattern). 1. Cysteic acid. 2. Aspartic acid. 3. Glutamic acid. 4. 3,4-Dihydroxyphenylalanine(?). 5. Glutamic acid (second spot). 6. Glycine. 7. Threonine. 8. Alanine. 9. Hydroxyproline. 10. Methionine sulphone. 10a. Methionine sulphoxide. 11. Histidine. 12. Arginine. 13. Lysine. 14. Unknown. 15. β-Alanine. 16. Proline. 17. Tyrosine. 18. γ-amino-*n*-butyric acid. 19. β-amino-*iso*-butyric acid. 20. Valine+methionine. 21. Unknown. 22. Phenylalanine. 23. Leucine+*iso* leucine. 24. Unknown. 25. Unknown. 26. Unknown.

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Plate 1. (a)

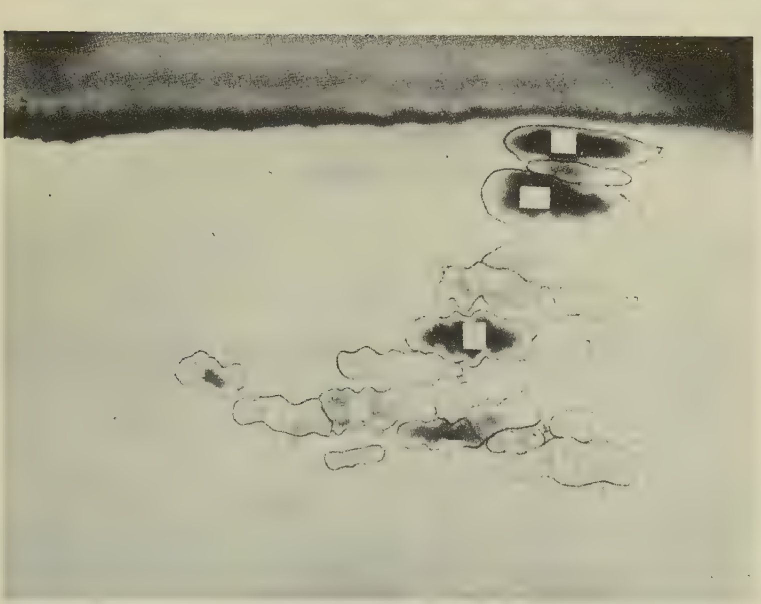


Plate 1. (b)

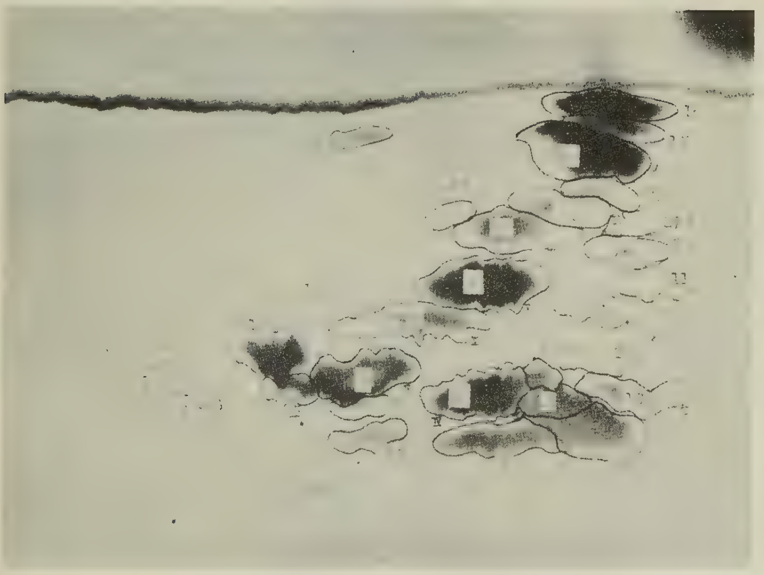
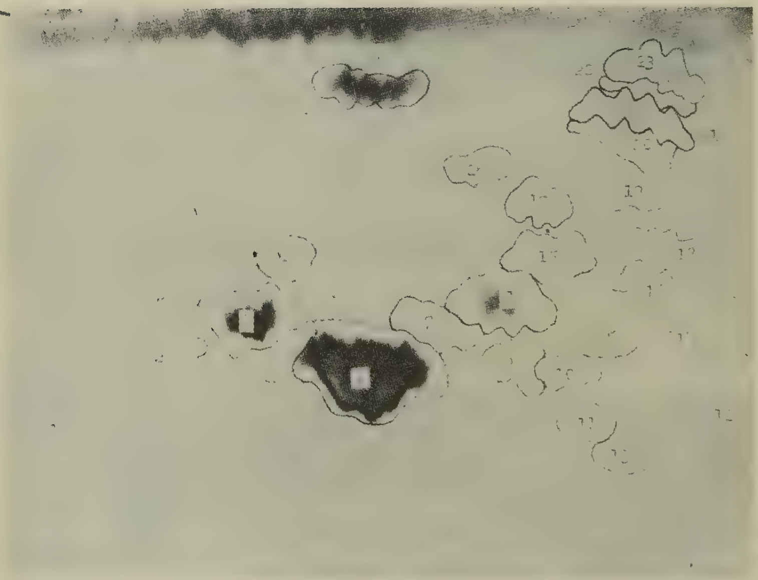


Plate 1. (c)



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A STRAIN OF *PASTEURELLA HAEMOLYTICA* ISOLATED FROM GOATS IN UGANDA

By

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INTRODUCTION

The object of this paper is to report the isolation of a strain of *Pasteurella haemolytica* associated with pneumonia and septicaemia in goats in Uganda.

Strains of *Past. multocida* of bovine origin were studied and classified by Jones (1921), who placed the haemolytic type in his Group 1. Since then, this group has been described in association with pneumonias, most commonly in sheep but also in adult cattle and calves; and with a septicaemia in lambs.

Tweed and Edington (1930) described a type of bovine pneumonia in England due to infection with a Pasteurella belonging to this group. Spray (1923) reported the isolation of organisms of this group from pneumonic lungs of sheep slaughtered in U.S.A. Newsom and Cross (1932) reported a similar organism from outbreaks of pneumonia in sheep also in the U.S.A., and suggested the name *Pasteurella haemolytica* for this group, but as it has not been shown that a true haemolysin is formed by this organism, Wilson and Miles (1955) do not recognise the subdivision. Montgomerie, Bosworth and Glover (1938) described a pneumonia of sheep in Wales in which a haemolytic Pasteurella was isolated from the lungs. In Scotland, Stamp, Watt and Thomlinson (1955) described an outbreak of septicaemia in lambs caused by this organism. An organism of the same group has also been isolated from the nasal cavities of normal sheep and cattle (Dungal, 1931; Francis, 1941; Bosworth and Lovell, 1944).

We have been unable to trace any reports of haemolytic Pasteurella associated with pneumonias in goats. However, Pande (1943) described an enzootic form of pleuropneumonia in goats, which occurred in India in which a Pasteurella strain was isolated resembling Jones' Group 2—i.e., non-haemolytic, non-capsulate, indole positive, and highly pathogenic to experimental animals. Borgman and Wilson (1955) described an outbreak of enzootic pneumonia in goats in U.S.A. in which a rather similar organism was isolated but it appeared less pathogenic to experimental animals, except mice. All the haemolytic strains with the exception of that described by Stamp *et al.* (1955) were non-pathogenic to sheep by experimental inoculation.

RESULTS

Isolation and Post Mortem Examination

Nine isolations of strains of haemolytic Pasteurella were made during routine bacteriological examination of specimens from goats that died at the Livestock Experimental Station, Entebbe. In each case, the post mortem examination and cultures from the specimens were carried out within twelve hours of death. The ages of the dead goats varied from one day to an adult of unknown age. The duration of clinical illness varied from less than twelve hours to several months.

The post mortem findings were similar in all cases. The animals were in good condition, except for one that had been ill for several months. The thoracic cavity showed the most obvious lesions, the lungs were congested and oedematous, the interlobular connective tissue being unusually prominent. In the less acute cases the apical and cardiac lobes usually showed some consolidation, and gangrenous pleuritis and pneumonia were present in two cases. The pericardial sac contained large amounts of clear fluid and the surface of the heart appeared congested. In four cases a large amount of clear pleural fluid was evident. The abdominal viscera were usually normal although in two cases the intestines were inflamed and tapeworms were present. The liver was pale. Further details of the isolations are given in Table 1.

TABLE 1

DETAILS OF THE ISOLATIONS

Post mortem No.	*Date*	*Age of goat*	*Duration of illness*	*Material cultured*	*Organisms isolated from:—*
270/58	August 1958	Adult of unknown age	2 weeks	Lung, brain, pericardial fluid, heart.	Lung, brain.
276/58	August 1958	3 days	less than 12 hours	,,	Lung
277/58	August 1958	4 days	,,	Lung, brain, pericardial fluid, heart, liver.	Lung, brain, pericardial fluid, heart, liver.
395/58	November 1958	2 mths.	1 day	Lung, brain, pericardial fluid, pleural fluid.	Lung, pericardial fluid, pleural fluid.
396/58	November 1958	3 mths.	,,	Lung, brain	Lung.
402/58	November 1958	9½ mths.	weak for several months	Lung	Lung.
129/59	March, 1959	4 mths.	less than 12 hours	Lung, pericardial fluid	Lung pericardial fluid
183/59	April, 1959	3½ mths.	1 day	Lung	Lung.
191/59	April, 1959	1 day	less than 12 hours	Lung liver	Lung. liver.

Bacteriology

Morphological and cultural characteristics. The organism is a gram negative short rod, with straight axis, convex sides and rounded ends, about 1·5 to 2 *u* in length, occurring singly and occasionally in pairs. It is pleomorphic and shows club, filamentous, navicular, giant, shadow and numerous other forms. It is non-motile at 37°C. and 25°C., and non-flagellate. On primary isolation it is heavily capsulated, but tends to lose the capsule on subculture. No endospores are produced. Bipolar staining is marked with weak stains. It is not acid fast.

Growth is good on ordinary media. Surface growth in 24 hours at 37°C. on blood agar shows circular, low, convex, smooth, glistening colonies, 1 mm. in diameter, with amorphous structure and entire, slightly bevelled edges. They are greyish in colour by reflected light, translucent, viscid on primary isolation, later becoming butyrous and easily emulsified. The colonies enlarge on prolonged incubation to 1·5 mm. in diameter at two days and are slightly umbonate, the central umbonate part being more opaque; later they become draughtsman-like and have a concave radially striated bevelled entire edge. At six days, the colonies are low convex and tend to lose their umbonate and draughtsman-like appearance, becoming smooth and up to 3 mm. in diameter, and have a distinct flat fringe. Growth on blood agar at 25°C. is also good although the colonies are smaller, being about 0·5 mm. in diameter after two days, 1 mm. after three days and slightly umbonate, and 2 mm. at six days, low, convex, smooth but with no fringe. On media containing bovine blood a narrow zone of partial haemolysis (alpha prime) is produced round each colony, and is most noticeable after twenty-four hours' incubation. On further incubation, the haemolytic zone does not enlarge and the colony overgrows it. However, the haemolysis is still visible and, on superficial examination, may give the appearance of extending beyond the edge of the colony due to the less opaque margin of the colony. The haemolysis is more marked at 37°C. than at 25°C.

On nutrient agar the colonies are similar to those on blood agar, but do not develop a fringe. On primary cultures on McConkey there is a good growth of small, pale pink colonies which only enlarge to 1 mm. in diameter after 6 days incubation. The ability to grow on this medium appears to be lost following subcultures on blood agar medium. There is a moderate confluent growth on nutrient agar stroke culture, filiform at the top and spreading at the bottom. The growth has a raised elevation, is contoured, moist, shiny with entire edge and is a grey colour by reflected light: it has no odour, is butyrous and easily emulsified. On gelatin stab culture, the degree of growth is moderate, filiform and extends the whole length of the needle track, with a little surface growth about 2 mm. in diameter round the puncture. There is no liquefaction of the medium. There is no growth on desoxycholate citrate agar medium, on potato medium, nor on Koser's citrate medium. Abundant growth occurs in nutrient broth, giving a uniform turbidity which later clears with a membranous or viscid deposit which does not disintegrate on shaking. On primary isolation there is a tendency for a thin pellicle to form.

Biochemical reactions. The organism ferments many sugars, giving acid but no gas in glucose, laevulose, galactose, sucrose, maltose, raffinose, xylose, arabinose (slight) and mannite. It does not ferment rhamnose, trehalose, inulin, dextrin, starch, glycerol, adonite, dulcite, salicin or inosite. Lactose and sorbite are variable. Litmus milk is unchanged or becomes slightly acid. Indole is negative, methyl red is negative, Voges-Proskauer is negative, and nitrates are not reduced to nitrates. Ammonia is not formed from peptone, there is slight hydrogen sulphide production, methylene blue is reduced, and catalase is strongly positive. There is no hydrolysis of urea. No soluble haemolysin is formed in a seventeen hour 10 per cent. serum broth culture.

Metabolic properties. It is an aerobe and facultative anaerobe and grows well at room temperature (24° to 25°C.) but better at 37°C. There is no growth at 5°C. The organism is sensitive to penicillin, using bacterial sensitivity test tablets of 2·5 IU, giving a zone of inhibition of 10 mm. diameter. On primary isolation, the organism dies out quickly at room temperature, usually being non-viable in three days.

Pathogenicity

Twenty-four hour broth cultures of strains 270, 276 and 277 were inoculated intraperitoneally into rabbits, mice, white rats and into goats about six months of age. The organisms were avirulent for all the animals except one guinea pig which was killed when moribund after two weeks and showed tubercle-like lesions in the spleen, liver and kidneys. The organism was recovered from the lesions. As a number of subcultures of these strains had been carried out prior to the animal inoculations, it was decided on isolation of a later strain (395) to carry out pathogenicity tests immediately. A mixture of a twenty-four hour broth culture (0.5 ml.) and ground up lung material was inoculated into three guinea pigs, three rabbits, nine mice and three white rats; and 1 ml. into two six-month old goats and two adult sheep, all by the intraperitoneal route. The goats were inoculated in addition with 1 ml. intravenously. Two guinea pigs and four mice died within twenty-four hours and the rest of the mice within a week, and the organism was recovered from the blood and organs of all of them. The remaining animals showed no signs of illness. Subsequently two young kids under twenty-four hours old were inoculated intraperitoneally with 2 ml. of an eighteen hour broth culture of the organism, and two other young kids, one under twenty-four hours and the other about forty-eight hours old, were given 1 ml. of the same culture intravenously. The three kids under twenty-four hours old died within twenty-four hours of inoculation whereas the forty-eight hour old kid survived. Haemolytic pasteurella were recovered from the blood and organs of the three that died.

DISCUSSION

It has been recognised for many years that haemolytic pasteurella can be isolated from pneumonic lungs of sheep, but it has been suspected that a filterable virus may be the initiating factor, the Pasteurella playing only a secondary role (Dungal, 1931; Newsom and Cross, 1932). Montgomerie *et al.* (1938) and Bosworth and

Lovell (1944), consider that the pulmonary invasion of the organism follows either the action of a virus or is the result of an environmental change. Stamp *et al.* (1955), on the other hand, consider that the strain isolated by them from lambs fulfills "Koch's postulates" as to the causal relationship to the disease as, unlike all the other strains described, their strain proved to be pathogenic to sheep by experimental inoculation.

Due to the very limited number of cases met with by us, it is difficult to assess the role this organism plays in the disease in goats. The low pathogenicity of the organism for experimental animals and especially to half grown goats by experimental inoculation would suggest that, in older animals at least, the organism plays a secondary role, possibly to a virus or to lowering of resistance caused by overcrowding and internal parasites. However, in two and probably three of the naturally occurring cases in very young kids, the organism was associated with a septicaemia which was umbilical in origin. Also on the same Experimental Station, a haemolytic Pasteurella identical to the strain from the goats was isolated in pure culture from the blood and organs of a two-day old calf that had died from navel ill. This evidence and the results obtained from the experimental inoculation of the day-old kids suggest that very young goats and other animals, particularly in the first twenty-four hours of life, may be much more susceptible than older ones to infection by this organism, and it may play a primary role in the disease. Colostral transmission of antibodies from the mother to the offspring may be the important factor in the acquisition of resistance to this organism in young kids.

CONCLUSIONS

Nine isolations of *Pasteurella haemolytica* from naturally occurring cases of pneumonia and septicaemia in goats in Uganda are reported. A description is given of the post mortem findings and the morphological, cultural, biochemical and pathogenic characteristics of the organism isolated. Experimental evidence is presented which suggests that very young kids may be considerably more susceptible than older goats to infection by this organism.

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SERUM PROTEINS OF *HIPPOPOTAMUS AMPHIBIUS* (LINNAEUS)

By

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INTRODUCTION

During June 1958 an experiment on the control of *Hippopotamus amphibius* (Linnaeus) was carried out in the Queen Elizabeth National Park, Uganda. This species (afterwards referred to as hippo) was at that time unusually numerous on the Kazinga Channel and was causing considerable destruction to vegetation with consequent soil erosion. During the experiment, all the hippo about the Mweya Peninsula were removed and, over a selected area on the Park shore of the channel, they were either shot or driven from wallows back into the channel. This afforded a unique opportunity for the collection of specimens by various scientific bodies. This communication deals with observations on the serum protein pattern of this species.

MATERIALS AND METHODS

All the animals from which blood specimens were obtained were killed by shooting through the brain, where possible on land but otherwise in the water, and later dragged ashore. In the latter case, it was not infrequently found that there had been some haemolysis in the interval between the death of the animal and the collection of the specimen. For the work referred to here, only non-haemolysed specimens were accepted and, of these, a hundred were examined. In most instances the blood was drawn from the axillary vessels following opening of the site with an axe and blunt dissection of the tissues. The blood was allowed to clot at the ambient air temperature after which it was kept on water ice until it reached the laboratory at Entebbe where the serum samples were separated.

Total serum protein estimations were made in all cases by the biuret method as described by King and Wootton (1956). A few were also made by the micro-Kjeldahl method. Paper electrophoresis was carried out according to the method of Flynn and de Mayo (1951) using a pH 8·6 veronal acetate buffer of ionic strength 0·1.

A quantity of 0·01 ml. of serum was placed on a strip of Whatman number 1 paper 36 cm. by 5 cm. and a current of 1·5 mA applied to each strip for eighteen hours. The paper strips were then stained with bromophenol blue, soaked in liquid paraffin, and read with a scanner (E.E.L.)*; the resultant curves were interpreted with the aid of a planimeter.

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RESULTS

It was noticed that though the serum samples were so pale as to be almost colourless they were considerably more viscous than sera from man or any other vertebrate previously studied by the author.** By the biuret method, the figures for total protein content were found to be unexpectedly high (mean 9·3 g. per cent), and there was a surprisingly low albumin-globulin ratio (0·52). To show that this was not due to an unknown substance in the serum which interfered with the biuret reaction, estimations on some samples were also carried out using the micro-Kjeldahl method and this gave identical results.

Electrophoresis showed the protein elements to be readily separable into four fractions. These correspond spatially to the albumin, and the alpha, beta, and gamma globulins seen in all species on which examination of this type has so far been reported.

TABLE 1

DATA FOR ONE HUNDRED HIPPO SERA

Relative percentages	*Albumin*	*Alpha globulin*	*Beta globulin*	*Gamma globulin*	*A/G ratio*	*Total serum protein g%*
Mean for 100 animals	34·5%	7·5%	17·2%	40·8%	0·52	9·3
Standard deviation . .	2·89	1·46	2·26	2·58	0·068	0·73
Maximum	40·3%	11·4%	23·2%	47·2%	0·68	11·4
Minimum	25·4%	4·6%	12·4%	34·7%	0·34	7·5
Mean for 100 animals in g% . .	3·2	0·7	1·6	3·8		

Table 1 summarises the findings, and from the results expressed as percentages it is apparent that there is little albumin, a very little alpha globulin and a reasonable level of beta globulin, but that there is a very large proportion of gamma globulin. When the figures are expressed as gramme per cent however, as shown in the bottom line of the table, the apparently small amount of albumin is really at the upper level of the expected limits. The greater part of the excess seen in the total protein figure lies in the gamma globulin fraction.

For ninety of the hundred specimens data relating to the sex and approximate age is available and is given in Table 2. It will be seen

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**Mr. S. D. Gangolli, Government Chemist, Uganda, was kind enough to estimate the viscosity of hippo sera which had been stored for some time at —20 °C, and to compare them with specimens of sheep serum which had been similarly treated. He found that the sheep sera gave an average figure of 1·6 in relation to water and the hippo sera 2·0.

that there were many more males than females in the sample taken. Table 3 shows the figures obtained from certain specimens selected to discriminate either between different age groups or the sexes.

TABLE 2

AGE AND SEX DISTRIBUTION OF NINETY ANIMALS

Approximate age	*Number of males*	*Number of females*
Very old	12	4
Adult	43	24
Young	5	2

TABLE 3

SERUM PROTEINS FOR NINETY ANIMALS ACCORDING TO AGE AND SEX

Relative percentages	*Albumin*	*Alpha globulin*	*Beta globulin*	*Gamma globulin*	*A/G ratio*	*Total serum protein g%*
Mean for 16 very old animals	33·7%	7·6%	17·9%	40·8%	0·53	9·5
Maximum	36·8%	10·9%	21·3%	44·2%	0·58	10·6
Minimum	25·4%	4·6%	13·8%	36·9%	0·34	8·8
Mean for 7 young animals ..	33·3%	7·6%	16·6%	42·0%	0·50	9·3
Maximum	37·4%	9·2%	20·3%	45·5%	0·60	10·3
Minimum	29·9%	4·6%	13·1%	37·9%	0·43	8·6
Mean for 43 adult males ..	34·8%	7·7%	16·5%	41·0%	0·54	9·4
Maximum	40·0%	11·4%	21·1%	47·2%	0·67	10·8
Minimum	26·5%	4·9%	12·7%	34·7%	0·36	7·5
Mean for 24 adult females .	34·4%	7·3%	17·8%	40·5%	0·53	9·2
Maximum	40·3%	9·5%	23·2%	44·9%	0·68	11·4
Minimum	29·6%	4·6%	12·4%	35·7%	0·42	8·2

The variation in the composition of the serum protein pattern does not occur with age or sex within the group examined.

It is generally considered that the closest relative to the hippo is the pig. For purposes of comparison, sera were collected from two domestic pigs of the Large White breed and from two bush pigs, *Potamochoerus porcus* (Linnaeus). The figures obtained on the four sera are shown in Table 4. The total protein levels fall within the range normally found in land mammals and none has the high concentration of gamma globulin demonstrated in the sample of hippo sera. The latter finding would, therefore, appear to be species specific.

TABLE 4

DATA OBTAINED FROM PIG SERA

Relative percentages	*Albumin*	*Alpha globulin*	*Beta globulin*	*Gamma globulin*	*A/G ratio*	*Total serum protein g%*
Two domestic pigs	29·5%	17·1%	21·5%	31·9%	0·38	7·0
	27·6%	20·0%	22·2%	30·3%	0·42	6·9
Two bush pigs	33·5%	21·2%	17·0%	28·3%	0·50	7·5
	30·3%	23·6%	17·9%	28·2%	0·43	7·5

DISCUSSION

The sample under consideration is drawn from a population which is predominantly male. It should also be pointed out that specimens from only a few young animals and none from infants were available, due to the fact that the majority of specimens came from the wallow area on the bank of the channel. Females were seldom encountered in the smaller wallows, whereas in the largest wallows, where there were sometimes numerous females, infants were rare. It would appear that the greater number of pregnant and nursing mothers remain in the channel, possibly for greater safety. As has been shown above, the composition of the population would not appear to be of importance when recording the protein pattern for the species, as breakdown by sex or age failed to yield significant differences.

CONCLUSIONS

Using the biuret method (confirmed by micro-Kjeldahl) it was found that the total protein in one hundred samples of hippo serum ranged from 7·5 to 11·4 g. per cent (Mean 9.3 g. per cent.).

Paper electrophoresis of the same series of sera revealed a curious distribution of protein fractions. There was a very low albumin-globulin ratio, the alpha globulin fraction was lower than expected, and there was a great excess of the gamma globulin fraction.

The high total protein figures appear to be due mainly to an absolute increase in the amount of gamma globulin.

Within the limits of the series, neither age nor sex influenced the serum protein pattern.

It was noted that hippo sera were very viscous when measured against sheep serum as a standard.

ACKNOWLEDGMENTS

The author would like to express her thanks to Dr. Maurice Weinbren who first suggested this study, to Mr. C. D. Juko of this organisation for carrying out the micro-Kjeldahl estimations and to all those persons concerned with the collection of the sera. It will be appreciated that a very great deal of effort went into the actual collection of the sera and it is regretted that all those who took part in it cannot be individually named.

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STUDIES IN IMMUNITY TO EXPERIMENTAL STAPHYLOCOCCAL MASTITIS IN THE GOAT AND COW

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INTRODUCTION

The high incidence of bovine staphylococcal mastitis in Great Britain and the difficulty in controlling this disease have emphasized the need for an evaluation of the degree of protection which might be conferred by prophylactic vaccination. The somewhat scanty literature on the use of vaccines and toxoids in the prophylaxis of mastitis in cattle has been reviewed in a recent paper by Pearson (1959), which suggests that more detailed information on the behaviour of the various cellular and extracellular antigens of the staphylococcus as immunising agents under controlled experimental conditions would be desirable. The scope of the present work was to examine antibody production in response to various staphylococcal vaccines, and to compare the degree of protection which these vaccines would confer against mastitis produced experimentally by the homologous strain of staphylococcus. On economic grounds, goats were used for the greater part of the experimental work since the goat has been shown to react to staphylococcal invasion of the udder in a similar manner to the cow (Derbyshire, 1958a, b).

MATERIALS AND METHODS

The general methods used are described below; the details of each experiment are included in the relevant section under "Results".

Preparation of Vaccines

Formolised cells. Staph. aureus strain 201 (Derbyshire, 1958a) was grown in Bacto-tryptose glucose glyceryl phosphate broth (Pattison and Matthews, 1957) for 24 hours, and the culture incubated for a further 24 hours after the addition of formalin to 0·5 per cent., when it was shown to be sterile. The cells were then separated by centrifuging, washed once in sterile isotonic saline and resuspended in saline to match opacity tube 10 of Brown's scale.

Adjuvant cells. These were prepared by adding 10 per cent. (w/v) aluminium hydroxide gel, made as described by Hektoen and Welker (1933), to formolised cells.

Formolised toxoid. Toxin was prepared by growing strain 201 in 0·3 per cent. nutrient agar for four days at 37°C under 25 per cent. carbon dioxide in air, and the agar removed by filtration through coarse paper. The toxin was sterilized by Seitz filtration, and tested for haemolytic activity by titrating dilutions in saline against equal volumes of 2 per cent. washed red cells of rabbit and sheep. It was then formalinised to 0·5 per cent., and held at 37°C, haemolytic tests being conducted daily to observe the progress of toxoiding, which was discontinued when a 1:5 dilution of toxin failed to lyse rabbit and sheep red cells. The toxoid was then stored at 4°C until used.

Cell-toxoid. Toxoid was added to packed formalin-killed cells to an opacity matching tube 10 on Brown's scale.

Adjuvant-toxoid. This was made by the addition of 10 per cent. (w/v) aluminium hydroxide gel to formolised toxoid.

Adjuvant cell-toxoid. 10 per cent. (w/v) aluminium hydroxide gel was added to the cell-toxoid prepared as described above.

Formolised whole culture. Strain 201 was grown as for the production of formolised cells, but after the addition of 0·5 per cent. formalin, the culture was held at 37°C for six days, by which time the culture was non-haemolytic at 1:5 for rabbit and sheep cells, in addition to being sterile.

Vaccination Procedure

The same procedure was adopted for each vaccine. Goats received a primary series of intramuscular injections of 2 ml., 5 ml. and 5 ml. of the appropriate vaccine, spaced two days apart, followed by a secondary injection of 5 ml. three weeks after the first primary injection. Freshly prepared vaccines were used for the secondary injection. In cows the doses of vaccine used were 5 ml., 10 ml. and 10 ml. for the primary injections, and 10 ml. for the secondary injection.

Tests for Antibodies

Blood serum samples were inactivated by heating in a water-bath at 56°C for 30 minutes and tested for staphylococcal antibodies as follows.

Agglutination test. A plate test was conducted on two-fold dilutions of serum in isotonic saline using a thick heat-killed suspension of strain 201 grown for 24 hours on nutrient agar.

Plate diffusion preciptin test. This was conducted as described by Pattison and Matthews (1957), using an acid extract of strain 201.

Antitoxin tests. Titrations of serum for alpha and beta antitoxin were carried out by a haemolytic test using Lh/10 doses of alpha and beta toxin prepared respectively from the Wood strain and strain 7428 of the National Collection of Type Cultures, with rabbit and sheep red cell suspensions as indicators. The toxins were standardized against alpha antitoxin obtained from the Medical Research Council Department of Biological Standards, and beta antitoxin from the Statens Seruminstitut, Copenhagen.

Free coagulase inhibitor. This was assayed by the method described by Duthie and Lorenz (1952), using coagulase prepared from strain 201.

Mouse protection test. Groups of ten 20 g. female White Swiss mice were inoculated intra-peritoneally with dilutions of Seitz-filtered inactivated serum in sterile saline, and 18 hours later each mouse was challenged by the intra-peritoneal inoculation of 0·5 ml. of a 24 hour culture of strain 201 in Bacto-tryptose glucose glyceryl phosphate broth. This test dose invariably killed all of the ten mice pre-injected with saline which were included in each test. The end-point for passive protection by a serum was taken as the highest dilution of serum at which at least five out of the ten mice survived the test dose during a seven-day observation period.

Method of Mammary Challenge Infection.

Goats were challenged by the inoculation into the teat canal of one half of the udder of 10 ml. of a whole 24-hour culture in Bacto-tryptose glucose glyceryl phosphate broth of strain 201, containing approximately 10^9 living staphylococci, as described in an earlier paper (Derbyshire, 1958a). The same technique was used to challenge cows, in which one quarter of the udder only was inoculated. Observations on the goats and cows were as described in the same paper, except that blood neutrophil counts and milk pH estimations were omitted. Histological examination of the goat udders was carried out as described by Derbyshire (1958b).

RESULTS

Antibody Response in Goats to Vaccination (Experiment I)

This experiment was designed to compare antibody production in the goat in response to the five staphylococcal vaccines listed in Table I. Each vaccine was tested in each of two one year old castrated male goats. Blood serum samples were obtained from each goat at weekly intervals for a period of 19 weeks from two weeks prior to the first vaccine injection.

In each goat, maximum levels of antibody occurred one week after the secondary injection of vaccine, and the levels attained at this time are shown in Table I, which also indicates the duration up to 14 weeks after vaccination for which the levels were elevated above the pre-vaccination titre. The agglutination reaction was not a reliable indication of the development of bacterial antibody; all goats had a titre of 1:20 to 1:80 prior to vaccination, and this was increased by all the vaccines tested, including toxoid only, by two or three tubes in the two-fold dilution series that was used. The gel diffusion precipitin reaction appeared to be a more reliable indication of the presence of bacterial antibody in the sera, but it had the disadvantage of being a qualitative test only. The antitoxin tests gave clear results; the addition of killed cells, and of cells plus adjuvant to the toxoid preparation increased the yield of antitoxin. The mouse protection test demonstrated highest levels of passive protection with serum from goats vaccinated with the adjuvant cell-toxoid, and there was a rough correlation between the mouse protective power of a serum and its antitoxin content. Coagulase inhibitor was not detected in the serum of any goat. It was clear that of this series of vaccines, the adjuvant cell-toxoid gave the highest and most persistent antibody levels. This vaccine was therefore made the subject of an intra-mammary challenge infection with the homologous strain 201, in Experiment II.

Resistance of Vaccinated Goats to Challenge (Experiment II)

Five one year old goats in the first month of their first lactation were vaccinated with the adjuvant cell-toxoid and five similar goats were used as non-vaccinated controls. Weekly antibody tests, with the exception of that for free coagulase inhibitor, were carried

TABLE I

ANTIBODY PRODUCTION IN EXPERIMENT I

Vaccine	Goat No.	*Antibody levels*											
		Agglutinins		*Gel diffusion*		*α antitoxin*		*β antitoxin*		*Coagulase inhibitor*		*Mouse protection*	
		Maximum titre	*Duration (weeks)*	*Maximum*	*Duration (weeks)*	*Maximum (units)*	*Duration (weeks)*	*Maximum (units)*	*Duration (weeks)*	*Maximum*	*Duration*	*Maximum titre*	*Duration (weeks)*
Formolised whole culture	74	2	8	—	—	16	5	8	8	Nil	—	1	3
	85	2	12	—	—	16	8	4	6	,,	—	2	7
Formolised cells	55	2	3	+	2	2	—	—	1	,,	—	Nil	—
	77	2	7	+	1	2	—	4	—	,,	—	,,	—
Formolised toxoid . .	59	2	14	—	—	8	9	16	8	,,	—	1	3
	60	2	14	—	—	32	7	8	7	,,	—	2	5
Cell-toxoid	50	3	7	+	12	64	14	64	10	,,	—	4	11
	56	3	14	+	5	64	9	16	9	,,	—	4	7
Adjuvant cell-toxoid . .	61	3	14	+	10	512	14	128	14	,,	—	8	14
	67	2	14	+	3	256	14	64	12	,,	—	8	14

The values for agglutinins indicate the rise in titre following vaccination expressed as number of tubes in the two-fold dilution series. The mouse protection titres are expressed as the reciprocal of the serum dilution which protected mice.

out on serum samples from both groups from two weeks prior to vaccination to the time of challenge, which was one week after the secondary injection of vaccine. Two of the vaccinated goats were slaughtered 15 weeks after infection for histological examination, and observations continued on the remaining three throughout the lactation.

Antibody production in the five vaccinated goats was similar to that observed in Experiment I, whilst the non-vaccinated group had consistently low antibody levels. Following the challenge infection, all of the five non-vaccinated goats developed gangrenous mastitis and were slaughtered on humane grounds as soon as the signs of gangrene were definitely detected. Mild mastitis, as described in a previous paper (Derbyshire, 1958a), occurred in all of the vaccinated goats, the characteristics of which were briefly as follows. A transient febrile reaction was observed in each goat six hours after inoculation, but all were clinically normal on the following day. The milk yield from the inoculated glands was reduced after infection, but in each goat had returned to its pre-inoculation level within four days. From one vaccinated goat, strain 201 was recovered from milk from the inoculated gland for only three days after infection, but organisms persisted in small numbers in milk from the inoculated glands of the other vaccinated goats. Milk neutrophil counts in the vaccinated group were elevated for from three to seven days after inoculation, after which only very small numbers of milk neutrophils were found until the end of lactation. No lesions were found on histological examination of one vaccinated goat which was slaughtered; in the other a few patchily distributed lesions containing areas of neutrophil migration characteristic of mild mastitis (Derbyshire, 1958b) were found. In this goat, only very small numbers of staphylococci were detected histologically and these were strictly confined to epithelial folds in the large ducts.

Protection of Cows with Adjuvant Cell-toxoid (Experiment III)

This experiment was designed to test the protective power of the strain 201 adjuvant cell-toxoid against homologous strain challenge in cows. Four two-year old Ayrshire cows in the first month of the first lactation were vaccinated and two similar cows were used as non-vaccinated controls. The challenge inoculation was made into the teat canal of the left hind quarter of all cows one week after the secondary vaccine injection. Observations were made on both groups for three weeks after challenge inoculation. Antibody levels were measured as in Experiment II.

Similar levels of antibody developed in the vaccinated cows to those which occurred in the previous experiments in goats vaccinated with adjuvant cell-toxoid. The reactions to challenge of the vaccinated and non-vaccinated cows are summarised in Table 2, from which it will be seen that the control cows developed severe, purulent staphylococcal mastitis with complete loss of milk secretion from the

TABLE 2

REACTION OF COWS IN EXPERIMENT III TO CHALLENGE INOCULATION

	Non-vaccinated cows (2)	*Vaccinated cows* (4)
Systemic reaction	Persistent (7 days)	Transient (12 hours)
Clinical udder abnormality	Persistent	Transient (12 hours)
Secretion of inoculated quarter:		
Macroscopic appearance	Sero-purulent exudate	Few clots in milk.
Bacterial count	Persistently high	High for 3 days.
Cell count	Persistently high	High for 4 days.
Milk yield of inoculated quarter	Negligible	Initial fall then steady increase.

TABLE 3

ANTIBODY LEVELS AND REACTION TO CHALLENGE OF GOATS IN EXPERIMENT IV

Vaccine	*Goat No.*	*Serum antibody levels*			*Reaction to challenge inoculation*
		α Antitoxin (units)	*Precipitin reaction*	*Mouse protection*	
Non-vaccinated	C371	0·5	—	0	Gangrenous mastitis
	C145	0·5	—	0	,, ,,
Formolised cells	C283	4	±	0	,, ,,
	C203	4	—	0	,, ,,
Adjuvant cells	C378	4	±	0	,, ,,
	C106	16	—	2	Mild mastitis
Formolised toxoid	C287	32	—	2	,, ,,
	C372	32	—	2	,, ,,
Adjuvant toxoid	C165	128	—	8	,, ,,
	C75	128	—	8	,, ,,
Cell-toxoid	C25	64	+	8	,, ,,
	C147	256	+	8	,, ,,
Adjuvant cell-toxoid	C288	128	+	8	,, ,,
	C109	256	+	8	,, ,,

The mouse protection titres are expressed as the reciprocal of the dilution of serum which protected mice.

inoculated quarter, whilst the vaccinated cows showed only a mild transient reaction. These cows continued to harbour strain 201 in the milk however, as did the vaccinated goats in Experiment II.

Nature of the Immunity Conferred by Vaccination (Experiment IV)

At this stage the opportunity arose to investigate the nature of the immunity produced by this vaccine in greater detail by testing the components of the adjuvant cell-toxoid, separately and in various combinations, for immunising power in goats. The preparations tested are listed in Table 3. Each vaccine was injected into each of two one year old lactating goats, and two non-vaccinated goats of similar age and stage of lactation were included as controls. Antibody measurements, method of challenge inoculation and post-inoculation observations on the goats were conducted as before. The experiment was terminated five weeks after inoculation, when the udders were examined histologically.

The serum antibody levels recorded in Table 3, are those at the time of challenge inoculation which was one week after the secondary injection of vaccine. Levels of β antitoxin closely paralleled those of α antitoxin. The non-vaccinated goat sera had low antitoxin levels, did not give a precipitin reaction and did not protect mice. The sera from the cell-vaccinated goats were similar, except that goat C106 had a relatively high natural antitoxin titre, and its serum protected mice at 1:2. Only trace reactions in gel diffusion precipitin tests were given by these sera. Formolised toxoid alone produced moderate antitoxic and mouse-protecting sera, which was enhanced by the addition of either killed bacterial cells or of adjuvant. Strong precipitin reactions were given only by sera from goats which had received cells in addition to toxoid. It will be seen from Table 3 that protection was only evident in those animals with moderate or high antitoxin levels. In these goats, the mild reaction to challenge was identical with that described for the vaccinated goats in Experiment II. It is clear that there was a correlation between serum antitoxin, mouse protecting power and immunity to mammary infection.

DISCUSSION

Minett (1939) has summarised experiments carried out by Bridré (1907, 1913), Klesov (1936) and Rosati and Bertolino (1938) who had used vaccines to prevent staphylococcal mastitis in the sheep with some evidence of success. Minett's own work showed that the use of toxoid in sheep prevented the systemic effects and, to some extent, the local effects resulting from inoculation of a mixture of staphylococcal culture and toxin into the teat canal. However, with one exception, all Minett's sheep were non-lactating and it could not be concluded that lactating animals would react in a similar manner. In the present paper, Experiments II and III have shown clearly that, under the experimental conditions employed, severe staphylococcal mastitis in goats and cows in full lactation can be prevented by vaccination with an adjuvant cell-toxoid prepared from the homologous strain prior to challenge by strain 201. How this immunity would be expressed under field conditions of exposure to staphylococcal infection remains to be seen, although in a recent paper Slanetz, Bartley and Allen (1959) described field experiments in which vaccination with a bacterin-toxoid prevented spread of infections and reduced the number of acute flare-ups in infected cows. Pearson (1959) described field observations made over a period of 2½ years in a dairy herd in which a vaccine/toxoid was used prophylactically against staphylococcal mastitis. During the first 18 months of the experiment there was a significant difference in the incidence of mastitis in treated and control groups, but not in the last 12 months of the experiment. This was interpreted as indicating that the reinforcing doses of vaccine/toxoid given every six months were insufficient to maintain immunity. The present paper has not provided information on the duration of immunity which the adjuvant cell-toxoid would produce, although in Experiment I, serum antibody levels were high for over three months in goats vaccinated with this preparation.

Two recent papers describe experimental work on immunisation of cows against staphylococcal mastitis. Spencer, Stewart and Lasmanis (1956) showed that the reaction in 16 quarters of cows vaccinated with a dried, formalinised staphylococcal culture suspended in aqueous aluminium hydroxide was less severe than the reaction in five quarters of non-vaccinated cows, all challenged with cultures of the homologous strain. Slanetz *et al* (1959) reported that four cows vaccinated with bacterin-toxoid showed a milder reaction than four non-vaccinated cows when challenged via the teat canal with 1 to 5 billion virulent mastitis staphylococci. In neither of these papers was it clear whether the immunity which was demonstrated was of an antibacterial or antitoxic nature since the vaccines used contained bacterial cells in addition to toxoid. In the present work Experiment IV, designed to provide information on this question, demonstrated a correlation between antitoxin content of serum and immunity to experimental mastitis, whilst there was no correlation between precipitin content of serum and immunity. Experiment I showed that the addition of killed bacterial cells and a physical adjuvant to toxoid increased the level and persistence of antitoxin production, confirming the finding of Ramon, Richou, Julienne and Jacquet (1953), who described a similar phenomenon in cattle. It is suggested that when the inclusion of formalin-killed cells in a toxoid vaccine enhances immunity, this may be due to an adjuvant effect on antitoxin production rather than to a stimulation of bacterial antibody production.

It may be significant that although only a transient inflammatory reaction occurred when the vaccinated goats and cows were challenged by the intramammary inoculation of massive numbers of Strain 201, the animals did continue to harbour this organism. However, this establishment of the infection in the milk duct system of the udder might not have occurred if the challenge dose of organisms had been lower, such as is more likely to occur under field conditions.

A further question of considerable practical significance is whether antitoxic immunity of this kind, produced by strain 201, would be as effective in increasing resistance to heterologous strains as it was shown to be against the homologous strain. Work at present in progress suggests that such may not be the case, and it must therefore be emphasized that the results reported here were obtained by vaccinating and challenging with the same strain of staphylococcus.

CONCLUSIONS

Serum staphylococcal antibody levels were higher and persisted longer in two goats vaccinated with *Staph. aureus* strain 201 formolised cell-toxoid with aluminium hydroxide gel added as an adjuvant than in pairs of goats vaccinated with simple formolised cell-toxoid, cells alone, toxoid alone or with a formolised whole broth culture.

The adjuvant cell-toxoid conferred a high level of protection in five goats against challenge by the intramammary inoculation of 10^9 living staphylococci of the homologous strain, the goats showing only a mild transient reaction compared with gangrenous mastitis in the controls. This vaccine conferred a similar degree of protection on four cows challenged in a similar manner.

A test of separate preparations of cells alone, toxoid alone, adjuvant cells, adjuvant toxoid, cell-toxoid and adjuvant cell-toxoid for immunising power against experimental mastitis showed that only those vaccines containing toxoid produced immunity.

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TESTICULAR NEOPLASMS IN DOGS

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INTRODUCTION

Testicular neoplasms are quite common in dogs. In a series of 2,361 tumours there were 160 (7 per cent) primary tumours of the male genital system and 136 of these, 85 per cent of those in that system, or 5·8 per cent of the total number of tumours, were testicular neoplasms (Cotchin, 1954). In this paper, an account is given of a series of testicular neoplasms, including these 136, from 318 affected dogs that have been examined in this Department.

MATERIALS AND METHODS

Sections of formalin-fixed material of testis tumours from 318 dogs, stained by haematoxylin and eosin, were available, and for selected tumours other methods such as van Gieson, Wilder's silver impregnation and Sudan IV were used. Taking as a guide the descriptions available in the literature, and particularly those of Innes (1942) and Harvey, Dawson and Innes (1940), it was possible to classify the testes as being affected with Sertoli-cell tumours (SCT), seminomas or interstitial-cell tumours (ICT), although it was not always easy to assign the neoplastic lesions unhesitatingly to one or other of these groups.

Most of the tumours were operation specimens, or were sent in for interest. From 33 of the dogs the affected testes were taken at autopsy in this Department: in 20 of these the testes were known to be enlarged, while the other 13 tumours were incidental post mortem findings.

RESULTS

Incidence of Different Tumours

Since in some dogs more than one tumour was present in one or both testes, to simplify description and to avoid duplication the affected dogs have been divided for convenience into three groups according to the only testis tumour present, or, where more than one type was present, according to the larger or largest of the tumours. Group 1, consisting of 157 dogs (49·4 per cent), comprised those animals in which Sertoli-cell tumours (SCT) were the only, or the most obvious, testis tumour present; Group 2, consisting of 107 dogs (33·6 per cent), comprised those with seminomas; and Group 3, consisting of 54 dogs (17·0 per cent), comprised those with interstitial-cell tumours (ICT) as the chief or only testis tumour. Thus, of every six affected dogs, three fell into group 1, two into group 2 and one into group 3.

Age incidence. The ages of 129 dogs in group 1 ranged from 3 to 15 years (average 8½), 89 in group 2 from 2 to 18 years (average 10), and 45 in group 3 from 5 to 16 years (average over 11½).

The average ages are in accord with those reported by Jones and Friedman (1950) and by Scully and Coffin (1952).

Author	*Average age of dogs with testis tumours (years)*		
	SCT	Seminoma	ICT
Jones and Friedman (1950)	7·6	11·1	12·1
Scully and Coffin (1952)	10	11	10
Present series	8½	10	11½

Breed incidence. Information was available about the breed, or lack of breeding, of 275 dogs only. Of these, mongrels and crossbreds, wire-haired Fox Terriers, Spaniels, Pekingese and Scottish Terriers formed the largest classes, the dogs being distributed in the three groups shown in Table 1.

TABLE 1

NUMBER OF DOGS OF VARIOUS BREEDS IN THE THREE TUMOUR GROUPS

Breed	*Number of dogs*			*Total*
	Group 1	*Group 2*	*Group 3*	
Mongrel and Crossbred ..	26	16	9	51
Wire-haired Fox Terrier ..	14	16	8	38
Spaniel	11	8	3	22
Pekingese	11	3	1	15
Scottish Terrier	7	10	0	17
Other breeds	69 (27*)	39 (16*)	24 (12*)	132
	138	92	45	275

* = Number of different breeds included in the total of "other breeds" in the group.

Site of Tumours

Information as to which testis was affected and whether the testis was in its normal situation in the scrotum or not was recorded for 209 of the 318 dogs. Of these, 84 (41 per cent) had tumours in ectopic testes, while the other 125 had tumours which were not specifically stated to be ectopic. Eighty had tumours of the right testis and 77 had tumours of the left, while 21 had tumours of both testes. The distribution of the tumours in the three groups of dogs is shown in Table 2. Thus, of 84 dogs with neoplastic ectopic testes, 65 were in group 1, 18 in group 2 and only one in group 3, forming 41·4, 16·8 and 1·9 per cent respectively of the total number of dogs in each group, or under 60, 30 and 4 per cent respectively of the dogs in the group for which information about the site of the affected testis was available. The usual situation of the ectopic testis was at or near the external inguinal ring, but in 10 dogs, 9 in group 1 and one in group 2, the testes were actually retained within the abdomen.

TABLE 2

SITUATION OF THE MAIN TESTIS TUMOUR IN THE THREE GROUPS OF DOGS

Site of main testis tumour	*Number of dogs*			*Total*
	Group 1 (SCT)	*Group 2 (Seminoma)*	*Group 3 (ICT)*	
Right testis	37 (19*)	33 (5*)	10 (1*)	80 (25*)
Left testis	40 (15*)	24 (7*)	13 (0*)	77 (22*)
Both testes	10 (6*)	6 (0*)	5 (0*)	21 (6*)
Ectopic, but side not stated ..	25 (25*)	6 (6*)	0 (0*)	31 (31*)
	112 (65*)	69 (18*)	28 (1*)	209 (84*)

*=Number of dogs in which neoplastic testis was ectopic.

Clinical Features

The chief clinical feature noted was an increase in size of the affected testis, but in 65 dogs (20 per cent) symptoms of hormonal disturbance were present. Alopecia was seen in 28 dogs (Fig. 1), 21 showed varying degrees of feminization, while a further 16 showed both alopecia and feminization. The relationship of these changes to the main tumour type present is shown in Table 3. Thus 45

TABLE 3

NUMBER OF DOGS IN EACH GROUP WHICH SHOWED ALOPECIA, FEMINIZATION, OR BOTH

Clinical feature	*Number of dogs*			*Total*
	Group 1 (157)	*Group 2 (107)*	*Group 3 (54)*	
Alopecia	17	2	9	28
Feminization	16	1	4	21
Both alopecia and feminization	12	1	3	16
	45 (28·7*)	4 (3·7*)	16 (29·6*)	65

*=Percentage of total number of dogs in the group.

(28·7 per cent) of the dogs in group 1, 4 (3·7 per cent) of dogs in group 2 and 15 (29·6 per cent) of dogs in group 3 showed alopecia or feminization or both.

The alopecia was generally rather bilaterally symmetrical in distribution although one side of the body might be more obviously affected. The hair was lost more or less quickly to form bald patches, the skin of which acquired a darker colour. In a few cases the bare skin appeared greasy or thickened. Favoured sites for the development of the alopecia were under the thorax and abdomen, inside the thighs, on the flanks and on the sides of the chest and neck, and near the root of the tail.

The feminization was shown partly by changes in behaviour and partly by changes in appearance. The chief sign noted was that the affected dog appeared to become as attractive to other male dogs as if it were a bitch in season. The penis often appeared small, and the prepuce flabby and pendulous. The teats became enlarged and sometimes showed a milk-like secretion. Some dogs were pot-bellied. The prostate gland, where available for examination, showed squamous metaplasia.

In dogs in group 1, there was sometimes a change of temperament not necessarily associated with obvious feminization: the dog might appear lethargic or apathetic, or alternatively it might become vicious or unusually aggressive. Some dogs became fat. Other signs were attributable to enlargement of the prostate, in one case accompanied by actual prostatitis. The non-neoplastic testis was generally noted to be unduly small In dogs in group 2 the chief sign was a painlessly enlarging and hardening testis, sometimes with a disporportionate enlargement of the scrotum. One retained neoplastic testis was detected when the abdomen was palpated to find the cause of obscure abdominal discomfort. In dogs in group 3, the tumours were often small and might not be certainly detected until the testis was incised.

Multiplicity of Tumour Types

Of the 157 dogs in group 1, three showed ICT in the same testis as the SCT, one showed a well-formed seminoma in the same testis as the SCT, four showed early seminomas in the affected testis, and one showed early seminoma in both the affected and the opposite testis. Of the six dogs with macroscopically obvious bilateral tumours, one (a 3-year old Pekingese) had bilateral retained testes, both with SCT; the others had bilateral SCT macroscopically, but in only 2 cases were both tumours examined histologically.

Of the 107 dogs in group 2, five showed SCT in the same testis as the seminoma, and one had ICT in the same testis. Of the 5 dogs in this group with bilateral macroscopic testis tumours, one had seminomas alone in both testes, two had seminomas in both testes, accompanied in one testis in each case by SCT; two dogs had a seminoma in one testis and a smaller ICT in the opposite testis. One dog in this group had a spindle-cell sarcoma of the scrotum.

Of the 54 dogs in group 3, both testes were affected by ICT in three. Early seminoma was seen in four dogs in the same testis as the ICT, and in two dogs in the opposite testis.

Clinical Duration

The tumours in dogs in group 1 had been noted for periods varying from 1 week to 3 years with an average of 5 months for 46 dogs; those in group 2 for periods varying from 3 days to 4 years with an average of 9 months, for 29 dogs, and those in group 3 for periods ranging from 7 days to 2 years with an average of 6 months, for 13 dogs.

Size of Tumour

The tumours in group 1 dogs varied from 1 to 15 cm. in length (average 5·1 cm.), in group 2 dogs from 1 to 11 cm. (average 6·3 cm.), and in group 3 dogs from 1 to 9 cm. (average 3 cm.).

Description of Tumours

Sertoli-cell Tumours

Macroscopically, these tumours were often bosselated structures with an intact tunica overlying them, and characteristically they showed on the cut surface a fine or coarse fibrous spongy network enclosing tissue which was white or light-grey, or occasionally creamy-white or even yellowish-ochre in colour. The tumours were usually firm in consistency. Small cysts, up to 1 cm. diameter or occasionally more, were sometimes present and contained a pale-brown fluid under pressure. The tumour masses, which were sometimes multiple and of varying size, tended to project above the level of the remaining testis tissue on the cut surface (Fig. 2). Sometimes the tumour tissue lying in the stromal sponge was soft, even pus-like, in consistency. Patchy yellow flecks of necrosis were sometimes seen. In one or two instances, the macroscopic appearance was confused with that of a seminoma.

The SCT, as is indicated by its alternative name of "Tubular adenoma", has essentially a tubular structure (Fig. 3), the tubules varying in diameter from 15 μ up to 1,000 or even 1,500 μ. They vary in diameter even in the same tumour, or in the course of the length of one tubule, but there is a tendency for the tubules to be of a fairly narrow range of size in any one tumour, or more particularly, in local parts of a tumour. Thus it is possible to recognize tumours, or parts of tumours, that are predominantly composed of relatively small tubules up to about 100 μ, medium-sized tubules 100 to 250 μ or so, and large tubules up to 500 μ or more. The small, medium or large tubules can sometimes be seen to be continuous. The smaller tubules may form as such, but in other cases they appear to be formed secondarily by fibrous septa growing into larger tubules. Some of the tubules can be seen to branch.

The fibrous tissue forming the walls of the tubules is variable in quantity and density. Sometimes it appears to be merely a somewhat thickened version of the normal tubular basement membrane, while in other instances it forms wider, more or less hyaline, sheets of fibrous tissue separating the tubular groups of cells. The fibrous tissue is usually rich in fibres and poor in cells, but occasionally it is more cellular; in one tumour it is very cellular and shows a number of mitoses.

The tubular arrangement of the cells appears to be an intrinsic property of the tumours and not an accidental conformation. It is not possible to explain the appearances as a simple sclerosis of the testis.

In the tubules, within whose limits they are generally confined, the tumour cells, if the tubule is of the order of 15 to 30 μ, may form single rows or solid cords, one or two cells thick, with no obvious lumen. The cells may stretch right across the tubules (Fig. 4) or form a single row near the fibrous tissue component leaving a lumen which usually has an ill-defined margin: sometimes there is a more clear-cut border to the lining cells giving a distinct columnar epithelial appearance.

In the rather larger tubules there is a tendency for a peripheral layer of cells to be orientated more or less perpendicularly to the fibrous wall and to be directed towards the centre of the tubule; in some instances, these cells stretch right across the tubule to give a palisading arrangement. This is one of the most characteristic features of SCT, but it is by no means constantly present and it may be developed to some extent in ICT.

In still larger tubules the centrally-placed cells are usually disorientated (Fig. 5), although sometimes they form a kind of streaming pattern. There may be intratubular papillae with vascular cores. In many, there is a central necrotic plug of tissue, or, less commonly, a pool of clear pink-staining fluid: cells at the edge of this fluid may appear to be under pressure. The necrotic plug can sometimes be seen to form from a degeneration of cells of a distinctly "sebaceous" appearance, with much finely vacuolated cytoplasm.

The tumour cells are variable in appearance, but at the periphery of the larger sheets of cells they are characteristically rather narrow and elongated with oval nuclei, while centrally they are more irregular with rounded nuclei. The cytoplasm is sometimes very like that of normal Sertoli cells with a fibrillar appearance. It is often ill-defined and forms an incomplete mesh around clear spaces, or shows more definite vacuolation: both these appearances are, in some instances at least, due to the presence of fat (Fig. 6). The vacuoles are single or multiple and may compress the nucleus to give a signet-ring appearance; occasionally, the vacuoles are very large, even reaching 60 μ. It is sometimes difficult to distinguish a fatty

SCT from a fatty ICT. Some of the cells contain a golden-brown granular pigment in the cytoplasm, or show larger globules of yellow pigment. A few cells contain rounded hyaline masses.

The nuclei show a fair range of size. Some isolated nuclei may be very large (one is 36 μ long) with large nucleoli, but generally the nuclei of the centrally-placed cells are pale and finely granular with a single rather small nucleolus. Some of the palisaded cells are rather large with pale nuclei; others are narrower with darker-staining nuclei. Transitional stages between these two forms can be seen. Mitotic figures are present in at least 46 of the SCT, but they usually occur only in very small numbers. In the typical SCT, nuclear abnormalities are exceptional, although in one tumour polynuclear cells are seen.

Difficulty is found in identifying 14 of these tumours simply as SCT. They show in part a much more richly cellular structure, with nuclear variations, mitoses and mitotic abnormalities that approach a typical seminoma in appearance. Although these seminoma-like tumours always show typical SCT areas and sometimes even show a smooth transition from typical SCT tissue to the seminoma-like tissue, it is not certain that these tumours are to be classed as merely unusually cellular and perhaps malignant SCT. It is possible that seminomatous transformation has actually occurred in certain areas, either from spermatogonial cells in original testis tubules surviving in the tumour area, or even from such cells included in SCT tubules during their development. In some parts of these tumours, isolated cells are scattered in the Sertoli-cell masses which, from their nuclear morphology, size, mitotic activity and nuclear abnormalities, appear to be scattered included seminoma cells (Fig. 7).

At the edge of the SCT there is usually no definite capsule, although there is considerable compression of surrounding testis tissue, and there is often a clear-cut boundary to the fibrous tissue of the tumour. The neighbouring atrophic tubules can frequently be seen to merge into the edge of the tumour and in many instances it seems that these surviving original testis tubules may actually be not only embedded in the SCT, but possibly incorporated into it as tumour tubules. These atrophic tubules may show Sertoli cells and spermatogonial cells in their lining epithelium.

Seminomas

Some seminomas filled the tunica which they bulged slightly and irregularly, but others showed a variable amount of surviving testis tissue of a rather brown colour when atrophied. This testis tissue often formed a narrow rim just beneath the tunica, or it might extend for varying distances between and around separate tumour masses when more than one of these were present in the same testis. The cut surface of the seminoma was usually homogeneous white or grey or pinkish-grey and typically showed no obvious coarse fibrous stroma, but sometimes a faint division into ill-defined lobules by fine fibrous strands was seen. Occasionally there was haemorrhage into the tumour tissue (Fig. 8). The seminoma tissue was often not surrounded by an obvious capsule, but, where one was present, it varied from 15 to 120 μ in thickness. The capsule appeared to form by compression of surviving testis tubules, although the smallest tumour nodule seen was surrounded by the thickest capsule.

Histologically, the characteristic appearance of a seminoma is of diffuse, or less frequently of more or less clearly intra-tubular, sheets of cells of varying size with large rounded nuclei. The nuclei often have granular chromatin and there is usually a prominent single (sometimes double) nucleolus. There is rather scanty cytoplasm, staining bluish-red or bluish in H. & E. sections. It may be clearly defined at the cell edge, or it may be ill-defined, and sometimes the cells seem to fuse together to form an apparent syncytium. There is a fairly wide variation in size of nuclei, some reaching 18 or even 30 μ in length; one nucleus for example is 18 μ long and contains a nucleolus 9 μ across. Polynuclear cells, with 2 to 5 or more nuclei, are seen. Mitotic figures are numerous and are quite often abnormal, forming, for example, tri-aster figures. Some of the unusually large cells have huge mitotic figures (Fig. 9). Karyorrhexis is often observed in the nuclei of scattered isolated cells or in small groups of cells, or there may be larger areas of necrosis. In one tumour there is a striking perivascular distribution of karyorrhectic nuclei.

The cells of the seminoma are generally rounded in shape, but some are a little elongated, approaching a short spindle shape. Occasionally vacuoles are seen in the cytoplasm and some cells contain yellowish-brown pigment granules. There is a variable, usually scanty, stroma of fibrous tissue, but in silver preparations there is a more obvious reticulin stroma which may have a rather narrow tubular patterning with a flag-like arrangement of tumour cells along the reticular fibres (Fig. 10). Occasionally, the collagenous stroma is more obvious and an "alveolar sarcoma" pattern is seen.

A characteristic feature of these tumours, commonly seen in seminomas and rarely seen in SCT or ICT, but also seen in the cellular lining of atrophic testis tubules, is the presence of a variable and often large number of rounded "clear spaces" in the tumour cell masses giving a "moth-eaten" appearance to the section. These clear spaces, which are 20 μ or more across, appear sometimes to form by vacuolation of an enlarged nucleus, the nucleolus sometimes being left in the clear space. Other clear spaces are formed by vacuolation of the cytoplasm of cells, some of which appear to have ingested other cells. Some clear spaces contain one or two peculiar shrunken cells, while others are apparently quite empty. These clear spaces do not appear to contain fat.

Perivascular foci of cells of lymphocyte appearance are present in at least 72 seminomas (Fig. 11). They are sometimes scanty, but sometimes very numerous. There is no correlation between their number and the size of the tumour except that perhaps the most marked accumulations are seen in and near some small seminomas. The surviving testis tissue only rarely shows spermatogenesis, but spermatozoa are seen mingled in the tumour cells in two seminomas. Scattered atrophic tubules may be embedded in the border of many seminomas and in some tumours these tubules lie deep in the substance.

In one dog, large cells with large nuclei are seen in the deeper part of the spermatogenic layer in tubules of the opposite, non-neoplastic testis and similar large cells, sometimes polynuclear or with very large nuclei, may be seen in the atrophic tubules outside some seminomas (Fig. 12).

Interstitial-cell Tumours

An arbitrary and possibly meaningless lower limit of size of 1 cm. was taken as distinguishing between ICT and hyperplasia of Leydig cells; generally, at about this size, a capsule was present, or forming, around the interstitial cell mass. The ICT were rounded or oval in shape. The size varied, but generally the tumours were rather small and there was at their edge a variable and sometimes extensive amount of testis tissue (Fig. 13). The tumours usually bulged quite prominently above the cut surface of the testis and showed a rather friable, sometimes haemorrhagic, tissue varying in colour from bright yellow, light or dull yellow-ochre, reddish-ochre or buff to salmon-pink. Sometimes, but less commonly, they were grey in colour. Some tumours showed smaller or larger cystic spaces filled with a mucoid or colloid material chiefly seen just inside the tumour capsule.

Histologically the cells are epithelial-like and arranged in mosaic, perithelial, petalloid or adrenal-like fashion, but in a few instances they form much longer ribbon-like cells arranged in bundles sometimes with a swirling pattern. The cells are supported by a varying and often rich vascular meshwork usually formed of thin-walled vessels. There is a tendency to haemorrhage and some haemosiderin is present lying in cells in the stroma. In one tumour there is a thrombus in a large vein.

The cells are sometimes arranged in rows or transverse layers in relation to blood-vessels, and a close approximation of the vessels at times gives an accidental appearance of a tubular arrangement of the cells: this may cause some confusion with SCT, but in the ICT the tubular patterning is less definite. In some places the tumour cells form solid sheets, branching cords or a rather cribriform mesh.

The nuclei, which tend to be placed in the part of the cell which is distal to the blood vessel near which the cell lies or to which it is applied, are of medium size, of the order of 9 to 12 μ long, oval or almost round in shape, moderately dense, sometimes with a fairly prominent nucleolus. In eleven tumours mitotic figures are present, usually in very small numbers. Some isolated nuclei are up to twice the normal size, but the general impression is of a rather regular and even size and shape of nuclei. The impression is also given that contact with or proximity to blood vessels appears to induce a columnar shape and distal polarity of the nucleus in these tumour cells (Fig. 14).

In eleven ICT, the nuclei of a varying number of cells contain opaque, homogeneous or slightly granular, faintly blue-staining hyaline bodies which in one instance can be seen to be distinct from the nucleolus (Fig. 15). These bodies are quite large and may fill half or more of the nucleus, or even bulge the nuclear membrane. In one nucleus, the nuclear body is surrounded by a clear space and closely resembles an "inclusion body". The cells have a fair amount of pink-staining cytoplasm which, because of its high fat content, is often finely foamy, or shows one or more vacuoles of varying size—even reaching 60 μ across (Fig. 16). In some cells, bright golden- or greenish-yellow pigment granules are present in the cytoplasm and in two tumours hyaline droplets of large size are present.

The capsule of the ICT is at least partly formed by compression of the surviving testis tissue and some atrophic tubules are incorporated into the capsule, or are embedded to varying depths in the substance of the tumour: the loss of spermatogenic activity in such tubules is not always total. The capsule varies in thickness from 15 to 500 μ, averaging 150 μ. The testis tissue around the tumour may show compression atrophy, particularly where it lies between the tumour and the tunica, but some of the tubules not under compression are also atrophic suggesting a possible hormonal effect.

In 18 testes, chiefly those containing the small tumours, the testis tubules show spermatogenesis. This is also seen in some tubules surrounded by ICT cells. In testes with larger tumours the tubules show varying degrees of atrophy: in the atrophic tubules occasional cells with very large nuclei are seen.

Malignancy

Only two dogs in group 1 are thought to have developed metastases from the testis tumour and for both dogs the records and material available are incomplete. One dog, an 8-year-old Spaniel, showed a large testis tumour with a small kidney lesion of similar structure. The second dog, a 6-year-old Pekingese, showed SCT in the intra-abdominal right testis. This tumour measured 6 × 4 × 4 cm. Histologically, a malignant tumour was found within the atrophic testis substance outside the capsule of this SCT and similar tissue was present in the liver and lungs in solitary foci. The histology of the malignant tissue in both these dogs is compatible with a diagnosis of malignant SCT.

Of the dogs in group 2 two showed metastasis of seminoma tissue. These were a 9-year-old Scottish Terrier with seminoma of the right testis and in the right deep inguinal node, and an 8-year-old crossbred Labrador with seminoma of the testis and iliac node. A third dog, a 14-year-old Bulldog with a seminoma of the left testis, is reported to have had metastases in the sublumbar nodes, but sections of these are not available. One of the dogs in group 3 showed metastasis. A 14-year-old fox terrier had an ICT, measuring $6 \times 4\frac{1}{2} \times 4\frac{1}{2}$ cm., in the right scrotal testis with extension into the spermatic cord. At autopsy 5 weeks after operation metastases were present in the inguinal and sublumbar lymph nodes and in the lung.

An attempt has been made to obtain follow-up histories for operation cases, but this has proved rather unrewarding owing to the conditions of veterinary practice. However, post-operative histories of a further 26 dogs in group 1, 13 in group 2 and 8 in group 3 covering periods of up to 8 years were obtained and gave no indication of metastasis in any of these dogs.

Hormonal Disturbances

The association of hormonal disturbances with testicular neoplasms in dogs has been known for a long time and, apart from its clinical importance, it has attracted interest because the production of hormone by the tumours might reflect the hormonal activity of the parent cell. SCT formation is often accompanied by alopecia or feminization, taken to be the signs of oestrogen production, and 45 examples were seen in this series. Removal of the affected testis was followed in at least 14 cases by reversal of alopecia and feminization, the speed of recovery sometimes being described by the owner as "startling".

In the cases of this present series where hormonal disturbances accompanied testis tumours which were not SCT, more details must be given. At the outset, it must be admitted that the term "alopecia" is much too broad to give a precise view of the type of baldness present. The work of Coffin and Munson (1953) in describing three different syndromes of alopecia in dogs may point the way.

Seminoma. There is no sure evidence in this series that seminomas produce oestrogen. Lack of information about follow-up histories in three of the four dogs with hormonal upset in this group means that it is not known whether removal of the neoplastic testis was followed by return to normal. The details of four cases are as follows.

(i) A poor section only is available of the tumour of the right testis of an 8-year-old crossbred Spaniel affected with dry skin and patchy loss of hair. The histology is of a seminoma, but the note on the macroscopic appearance of the tumour suggests SCT: confusion of case material may have occurred.

(ii) An undoubted seminoma was present in the right "undescended" testis of a 10-year-old mongrel, which "had always been attractive to male dogs". The neoplasm had been noted for 2 years.

(iii) The right testis of an 11½-year-old wire-haired Fox Terrier with alopecia and sex inversion showed a seminoma.

The following case shows how confusion might arise in the absence of post-operative histories.

(iv) A 6-year-old mongrel had bilaterally symmetrical alopecia of gradual onset and about 6 months' duration which did not respond to treatment with thyroid extract. The left testis, which was firmer and slightly larger than the right, was removed. It measured 2·2 × 2·0 cm., and was almost entirely composed of seminoma tissue. No spermatogenesis was seen in surviving testis tubules and some of these showed early seminoma formation. No improvement of the coat followed operation and a series of testosterone implants having been tried without effect on the coat, the right testis was removed 18 months after the previous operation. It measured 3 × 2 cm. and was largely occupied by SCT. Hair growth was good 2 months later following this second operation, and has remained so for 5 years. In this dog, it seems fairly certain that the alopecia was attributable to the SCT. Seminoma formation in the left testis may actually have developed later than the SCT, but may have outgrown it.

Interstitial-cell tumours. The cases in this group, in which alopecia and feminization occurred, are summarized in Table 4. The lack of complete post-operative histories reduces the value of the facts recorded in Table 4, but it can be said that ICT, generally of small size, may be found in the testis of dogs with alopecia or feminization. In three cases (A.31, 38, 7709/58) and to a lesser extent in a fourth (A.17) it is suggested that the post-operative recovery may indicate an oestrogenic effect of the tumours.

Kahan (1955) reported skin troubles, including alopecia, in a 7-year-old Brittany Spaniel which developed feminization. A nodule of ICT, one cm. in diameter, was found in the right testis. The dog was castrated (presumably both testes were removed) and recovery occurred. Kahan refers to a reported case of gynaecomastia accompanying ICT in a 32-year-old man.

The case reported by Laufer and Sulman (1956) is also suggestive that ICT in dogs may be oestrogenic, although in a later note (Editorial, 1957) "the authors wish to state that the data regarding the urinary excretion of oestriol, oestrone and oestradiol are not to be regarded as definitive and may be disregarded". These authors reported finding a tumour of the left testis of a 7-year-old Boxer which was not examined histologically. The Allen-Doisy test using castrated female mice gave no evidence of oestrogenic hormone in 2 g. of testicular tissue, but the tumour contained 1,000 I.U. of oestrogenic hormone. The feminization and alopecia previously noted persisted and later ataxia developed. At autopsy after destruction, the right testis showed multiple nodules of ICT and similar tissue was present in the right lobe of the cerebellum. In the right testis and brain tumour tissue there was present lipoid material, staining with Sudan III, not doubly refractile and Ashbel-Seligman negative.

DISCUSSION

In accordance with the excellent studies of Innes (1942), most

TABLE 4

Case No.	Age (years)	Breed	Neoplasm		Symptoms	Result of removal of neoplastic testis
			Site	Size (cm)		
Alopecia						
A.13	14	W.H.F.T.	Left	?	Alopecia for several months. No response to thyroid	Not known
A.29	12	Labrador-cross	Left	(3×2)	Alopecia both flanks. Also a mixed mammary tumour	Not known
A.31	9	Cairn Terrier	?	(1)	Very severe alopecia. Other testis a "fibrous node"	Hair growth in 9 months. Both testes removed
A.38	13	Terrier		(4×3) (2×1)	Almost complete alopecia for 2 to 3 years	Both testes removed. Good coat 6 months later
C.82	13	Red Setter	Right	(1·5×1)	Bilateral symmetrical alopecia	Not known. Early seminoma in left testis
2906/58	Aged	W.H.F.T.	Right	4×2½×3	Slight alopecia	
6710/57	11	Golden Labrador	Left	4×3×3 (3×3)	Alopecia	
7709/58	13	Cairn T.	Left	2·2×1·7 (1 cm)	Alopecia	Hair regrown 10 weeks later
10602/58	9½	English Setter	Left	3·7×3·0×3·0 (1·0×0·7)	Alopecia	
Feminization						
A.11	5	Mongrel	"semi-retained"	(1)		Not known
A.17	8	W.H.F.T.	?	3×2 (2×1·5)	Slight sex inversion	Destroyed 9 months later. Inversion not then noted
12304/59	13½	Terrier-X	Left	2·6×1·8 (1·5×1·0)	Feminization	
3304/59	12	English Setter	Right	2·5×2·5×4 (2·5×2·5×2·5)	Feminization	
Alopecia and feminization						
A.5	8	W.H.F.T.	?	(1)	Loss of coat. Teats and prostate enlarged	Not known (both testes removed)
A.14	12	W.H.F.T.		(6×4×3)	One year—loss of hair, enlarged teats, attractive to other male dogs	Not known
18810/58	13	Mongrel	Right	2·4×2 (1, 0·7)	Alopecia, gynaecomastia (squamous metaplasia, prostate)	Destroyed after street accident

observers are now agreed that the testicular tumours of dogs can be classified in general as Sertoli-cell tumours (sustentacular-cell tumours, tubular adenomas), seminomas, or interstitial-cell tumours (Leydig-cell tumours). Good descriptions of these types have been given by many authors including Schlotthauer, McDonald and Bollman (1938), Huggins and Pazos (1945), Mulligan (1949), Jones and Friedman (1950), Bloom (1954), Willis (1953) and Scully and Coffin (1952). Schlotthauer *et al.* (1938) included Sertoli cell tumours under the name of adeno-carcinomas. The rarity and perhaps absence of testicular teratoma or chorionepithelioma in dogs is a striking and unexplained fact. The findings in this present series of tumours are in general agreement with those of other workers, but some comments can be made on certain aspects of the individual tumours.

Presumed early stages of SCT are rarely encountered (Scully and Coffin, 1952). In the present series, it seems possible that local atrophic tubules were not merely enclosed within the tumour capsule and the tumour substance, but were actually being transformed into tumour tubules. In this connection, attention may be drawn to a feature of SCT which was illustrated by Scully and Coffin in Figs. 10 and 12 of their paper—the occurrence in the actual neoplastic SCT of cells which may be surviving spermatogonia and which may be transformed into seminoma cells.

The studies of such workers as Schlotthauer *et al.* (1938), Mulligan (1949), Scully and Coffin (1952) and Willis (1953) support the view that the seminoma of the dog develops from cells of the spermatogonial series and that the origin is often diffuse, multifocal and intrinsically intratubular. The details of the neoplastic transformation and its relation to hyperplastic or hypertrophic changes in spermatogonia mentioned by Sommers (1956) need further study. Spermatogenesis is generally suppressed in tubules in which seminoma is forming, but not necessarily in the whole testis. The tendency for seminoma to develop in atrophic testis tubules outside a larger tumour, whether seminoma, SCT, or ICT, has been noted.

It is of interest that ICT does not often seem to affect the ectopic testis—Schlotthauer *et al.* (1938) found none in the "cryptorchid" testis—although Leydig cells are prominent in retained abdominal testes.

The relative incidence of the three main types of testicular tumour in dogs is rather variably reported, and the present series is the only large one in which Sertoli-cell tumours were the most numerous type. Bloom (1954) stated that SCT are less common than seminomas, as did Scully and Coffin (1952). Salerno (1956), in a series of testis tumours from 13 dogs, found them, in order of frequency, to consist of Sertoli-, germ-, and interstitial-cell tumours. Some of the other reported series (Schlotthauer *et al.*, 1938; Huggins and Pazos, 1945; Scully and Coffin, 1952) contain a predominant number of ICT; these are often small tumours and are thus more easily found incidentally on cutting into a testis at autopsy. In the present series the tumours were almost entirely operation specimens and the larger the tumour, the more easily it would attract the owner's attention.

In respect of age incidence in our series, and more obviously in that of Jones and Friedman (1950), there is perhaps a tendency for SCT to be observed in dogs rather younger than those with other types of testis tumour. If so, it may be that this is related to the rather frequent association of such tumours with ectopia of the testis, or to the common accompanying hormone disturbances, both of which may attract attention earlier to the possibility of a testis neoplasm being present.

At the present time, it is not possible to give a significant analysis of the relative breed incidence in this series of testis tumours, as the composition of the population from which the affected dogs came is not known and information about the affected dogs is incomplete.

According to Brodey and Martin (1958) in 31 (of 40) dogs with SCT for which information was available, the right testis was involved in 22, the left in 5 and both in 4. Brodey and Martin state that they have observed that right-sided cryptorchidism is much commoner in dogs than left-sided. They further state that since cryptorchidism favours neoplasia, an assumption that needs testing, there may be a direct relationship between the high incidence of right-sided neoplasia and cryptorchidism.

In this series, however, there seems to be no tendency for one testis to be affected more frequently than the other.

It was found that 21 dogs out of 209 for which information was known had tumours in both testes, and in accord with other observations (Bloom, 1954) bilateral orchidectomy should be considered in cases where one testis only is clinically involved.

In 84 dogs out of 209 the neoplastic testis was ectopic. The tendency for testis neoplasms, when they do occur, to be seen in ectopic testes, is also a feature in man (Campbell, 1942; Anderson, 1949; Homburger and Fishman, 1953; Evans, 1956). Greulich and Burford (1936), who described SCT in the abdominal testes of 3 dogs, one being a bilateral abdominal cryptorchid, pointed out that the occurrence of unilateral cryptorchidism would indicate some defect in the ectopic testis itself which rendered it unable to respond properly to whatever stimuli normally direct the course of testis descent. Mann (1956), in a very useful account of cryptorchidism in dogs and other species, states that the hypophysis is suspected of activating the process of descent through the agency of male hormone.

CONCLUSIONS

An account is given of testicular neoplasms in 318 dogs. Sertoli-cell tumours were the most numerous, forming the largest, or the only, testis tumour in 157 dogs, followed by seminoma (107 dogs)

and interstitial-cell tumours (54 dogs). The macroscopic and histological features of the tumours are described. Reference is made to the age and breed of the affected animals, and to the association of tumour formation with (a) ectopia testis, seen in 84 of the affected dogs, and (b) endocrine disturbances, characterized by alopecia and/or feminization, seen in 65 dogs. The occurrence of multiple tumours and mixed tumour types is noted. In only 6 dogs were metastases known or suspected.

The findings are discussed in relation to reports by others.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Sertoli-cell tumour of left testis of 12-year-old Alsatian dog showing oedema of prepuce, enlargement of teats and alopecia inside thighs.

Fig. 2. Same case as Fig. 1. Sertoli-cell tumour of testis showing lobulated appearance, with coarse fibrous mesh and bulging masses of tumour tissue. (Scale in cm.).

Fig. 3. Sertoli-cell tumour of ectopic testis of 10-year-old wire-haired Fox Terrier, showing tubular structure. × 76.

Fig. 4. Sertoli-cell tumour showing tubular structure. Cells with clear cytoplasm and cells stretching across width of tubules in a palisaded fashion. × 348.

Fig. 5. Sertoli-cell tumour of ectopic testis of 10-year-old mongrel dog showing disorientated arrangement of cells in a broad tubule, with some indication of radial arrangement at periphery (lower border). × 230.

Fig. 6. Sertoli-cell tumour of 9-year-old Airedale showing vacuolation of tumour cells which have contracted away from wall of tubule. × 348.

Fig. 7. Sertoli-cell tumour of dog (no clinical data available) showing a multinucleated cell in a tubule—possibly an included seminoma cell. × 348.

Fig. 8. Seminoma of left testis of 12-year-old mongrel dog showing tumour filling intact tunic and faint lobulation; there is haemorrhage in one part. (Scale in cm.).

Fig. 9. Seminoma of right testis of 10-year-old Scottish Terrier (both testes were affected) showing large single nuclei (upper right), multinucleate cell (bottom right), mitosis (top left), large mitotic figure (middle left) and "clear space" (bottom left). × 340.

Fig. 10. Seminoma of testis of 18-year-old Wire-haired Fox Terrier (other testis showed interstitial-cell tumour) showing nuclear conformation, mitotic figure, scanty cytoplasm and a flag-like arrangement of tumour cells along fine septa. × 760.

Fig. 11. Seminoma of testis of 10-year-old Pekingese showing lymphocyte accumulation near blood vessels (bottom right) and focus of degenerating cells (top left). × 220.

Fig. 12. Atrophic testis of 6-year-old mongrel dog showing two large cells with large nuclei and large nucleoli in atrophic spermatogenic epithelium, possibly early seminoma formation. × 760.

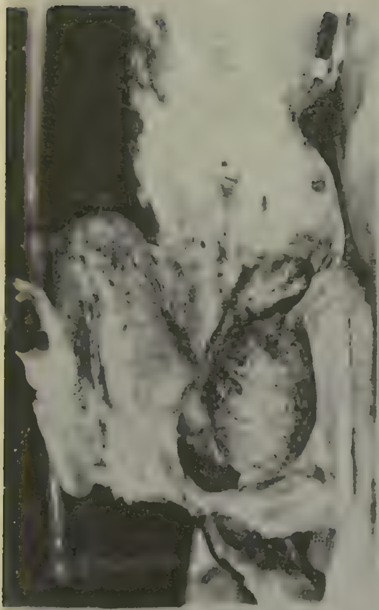
Fig. 13. Interstitial-cell tumour of testis of dog (no clinical data available) showing non-fibrous bulging tumour which was a yellow-ochre colour. (Scale in mm.).

Fig. 14. Interstitial-cell tumour of 12-year-old Spaniel showing distal position of nuclei of epithelium-like cells in relation to a blood vessel. × 348.

Fig. 15. Interstitial-cell tumour of testis of 10-year-old Miniature Poodle showing homogeneous intranuclear structures in some tumour cells. × 760.

Fig. 16. Interstitial-cell tumour of 12-year-old mongrel dog showing marked vacuolation of tumour cells. × 348.

All sections stained H. & E.



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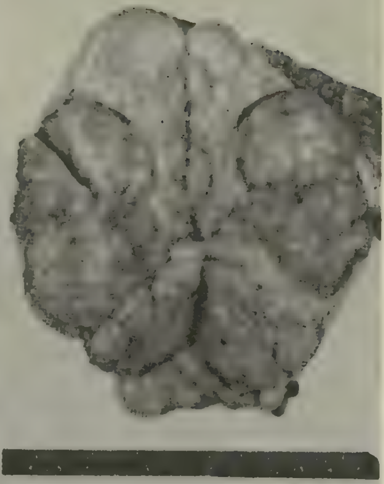

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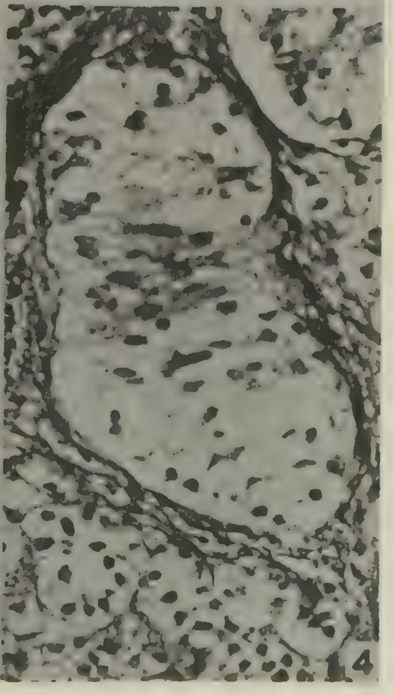

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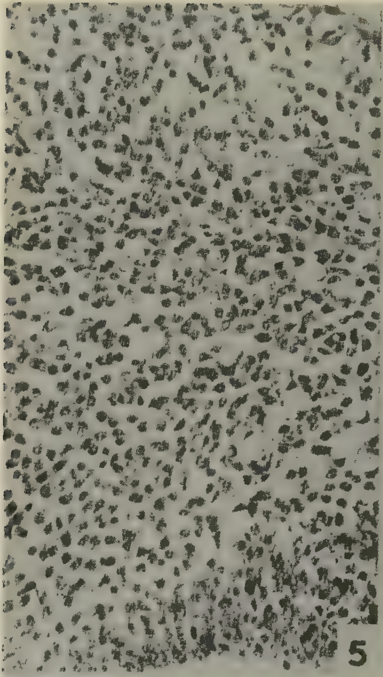
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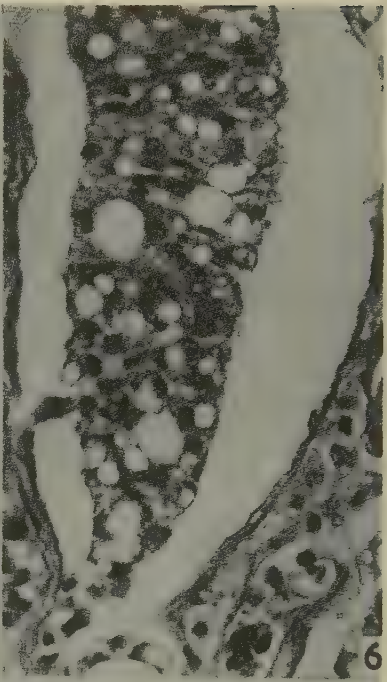
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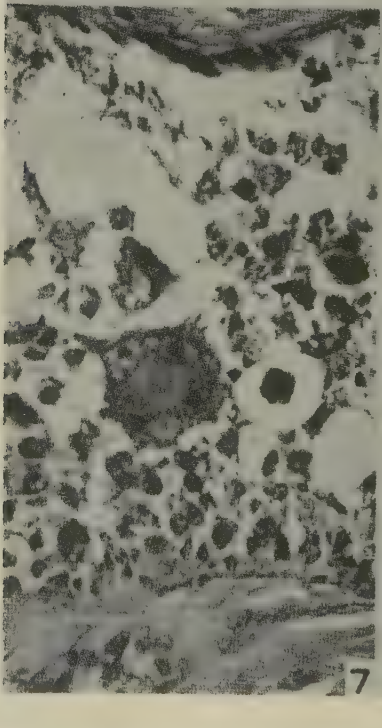
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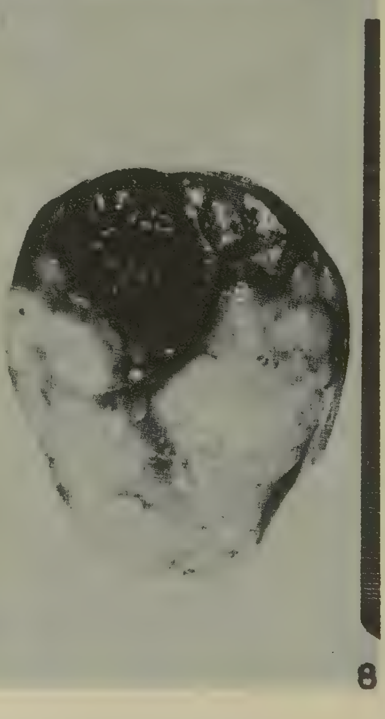
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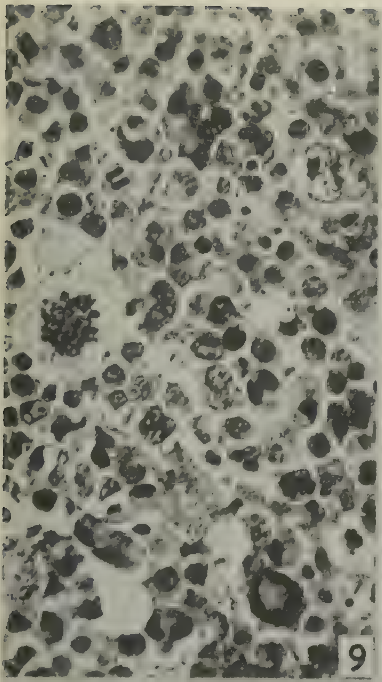
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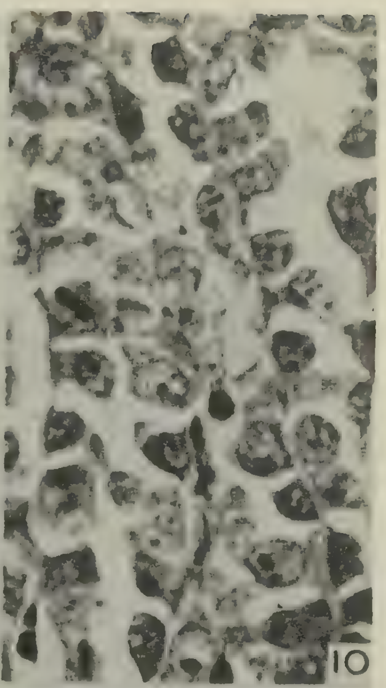
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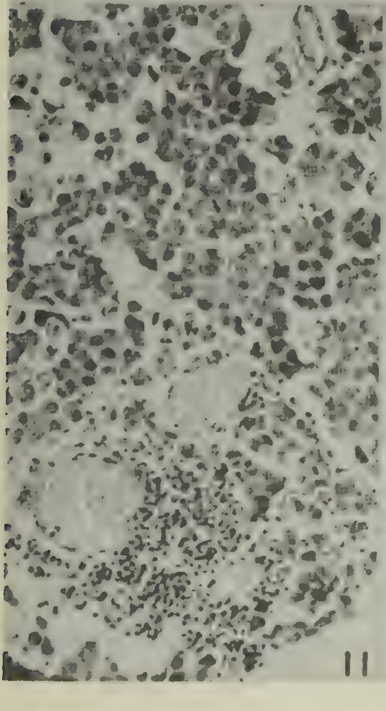
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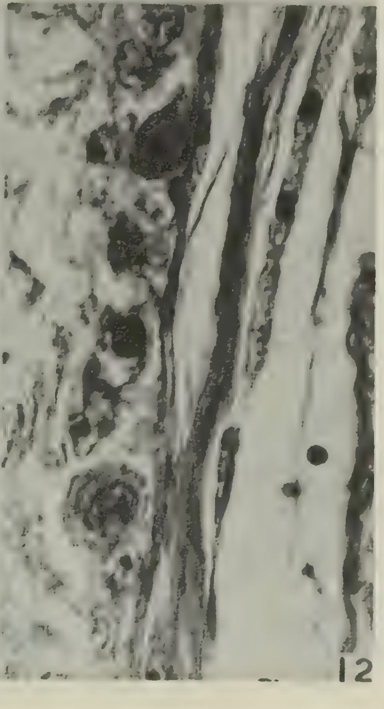
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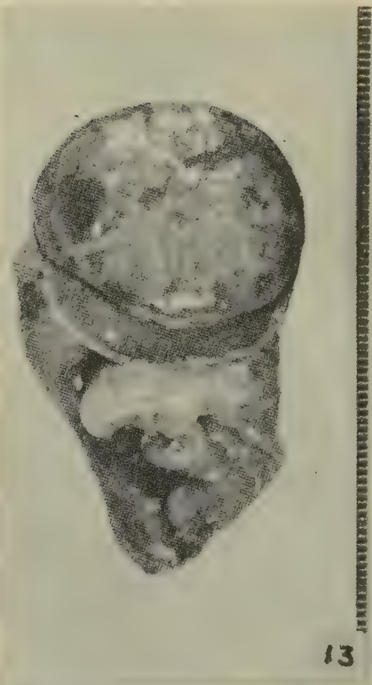

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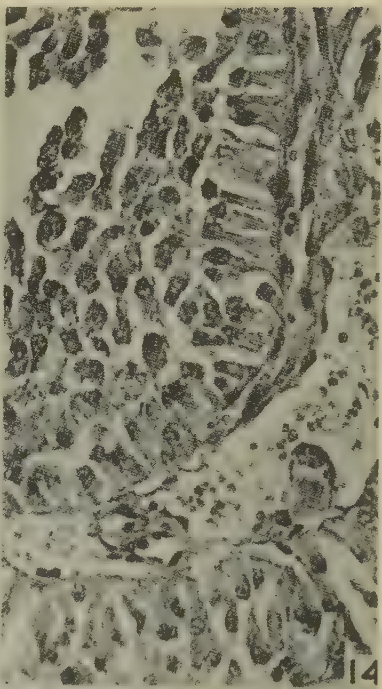

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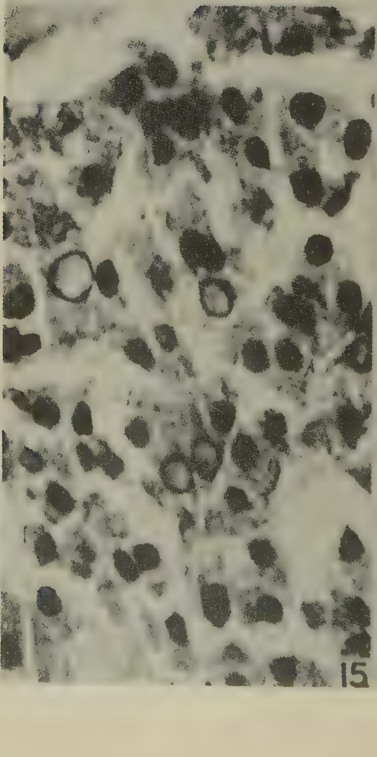

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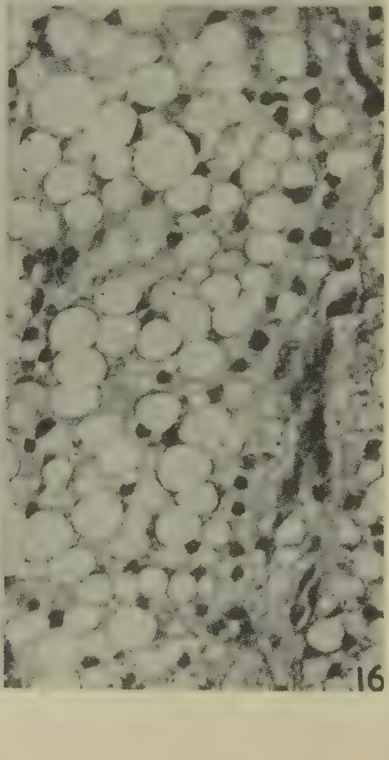

16

ASSOCIATIONS OF VARIOUS SULPHONAMIDES AND PYRIMETHAMINE IN THE CHEMOTHERAPY OF CAECAL COCCIDIOSIS*

By

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INTRODUCTION

Lux (1954), while testing a series of 2:4-diaminopyrimidines for activity against *Eimeria tenella* in chicks, noted synergism between pyrimethamine (2:4-diamino-5-p-chlorophenyl-6-ethylpyrimidine) and two sulphonamides, sulphamerazine and sulphanilamide. Kendall and Joyner (1956a, b) showed that the anticoccidial activity of sulphadimidine was potentiated by pyrimethamine, and found that certain mixtures (sulphadimidine 0·05 per cent w/w plus pyrimethamine 0·005 or 0·0025 per cent w/w) fed from the second to the fifth day following infection with *E. tenella* oöcysts gave good control. By contrast, when medication was commenced before infection (Joyner and Kendall, 1956) or if higher concentrations of both drugs were used in combination (Kendall and Joyner, 1956b), there was little therapeutic effect although deaths were delayed in comparison with the untreated controls. Marthedal and Velling (1957) reported that six- or seven-day treatments with 0·01 per cent sulphaquinoxaline mixed with 0·0025 per cent pyrimethamine, and 0·05 per cent sulphadimidine plus 0·005 or 0·0025 per cent pyrimethamine which started within a day following infection did not give full control, as symptoms of acute caecal coccidiosis usually occurred 4 to 5 days after withdrawal of the drugs. On the other hand, Arundel (1957) states that delayed deaths did not occur after medication with 0·01 per cent pyrimethamine added to sulphaguanidine, sulphaquinoxaline, sulphadimidine, sulphamerazine and sulphapyrazine each at one-fifth of their normal concentrations, but the duration of the treatments and the time they commenced relative to infection is not given. The increased anticoccidial action of sulphaquinoxaline and sulphaguanidine by the addition of pyrimethamine was briefly reported by Kendall and Joyner (1956a).

Our interest in the chemotherapy of coccidiosis led us to compare the activities of sulphadimidine and sulphaquinoxaline, each mixed with pyrimethamine against experimental *E. tenella* infections. With the exception of sulphapyrazine the other sulphonamide-pyrimethamine associations mentioned above as well as sulphadiazine, sulphapyridine, sulphathiazole, succinylsulphathiazole and phthalylsulphathiazole, so far not reported in combination with pyrimethamine, were also examined to observe the effect of courses of medication that commenced before infection, and the results are given in this paper.

METHODS

Six- or 7-day old Rhode Island Red x Light Sussex cockerels, obtained

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*The majority of this work formed part of a thesis for the M.Sc. degree of the University of London.

TABLE I

EFFECT OF SULPHONAMIDES ALONE AND IN COMBINATION WITH PYRIMETHAMINE WHEN FED TO CHICKS FOR 8 DAYS COMMENCING 24 HOURS BEFORE INFECTION WITH *E. TENELLA* OÖCYSTS

Sulphonamide	*Sulphonamide alone*			*Sulphonamide plus 0·005% pyrimethamine*			*Mortality* among control groups by the 7th day after infection*			
	% in food	*Mortality:* days after infection*		*% Sulphonamide in food*	*Mortality:* days after infection*		*Pyrimethamine*		*0·05% Sulphaquinoxaline*	*Infected untreated controls*
		7th	*13th*		*7th*	*13th*	*0·005%*	*0·01%*		
Sulphanilamide†	0·5 0·25	16/21 11/11	16/21	0·5 0·25 0·125	1/10 2/11 6/11	7/10 7/11 6/11	19/21		0/11	20/21
Sulphadiazine	0·1	9/11	9/11	0·01	0/11‡	6/11	9/10	4/10		12/12
Sulphaguanidine**	0·5 0·25	7/21 22/32	8/21 22/32	0·25 0·125 0·06 0·03	0/21 0/11 0/10 0/10‡	9/21 4/11 1/10 0/10	19/21		1/21	32/34
Sulphamerazine	0·1 0·05	5/10 8/10	5/10 8/10	0·05 0·025	2/10 10/10	5/10	9/10		1/10	11/11
Sulphapyridine	1·0	10/10		0·5	7/8		8/10		1/10	11/11
Sulphathiazole†	0·5 0·25	9/10 17/20	9/20 17/20	0·125 0·06	0/9 7/10	6/9 7/10	8/10		1/10	20/21
Succinylsulphathiazole†	0·5	6/20	6/20	0·5	4/20	4/20	7/10	0/10	1/20	10/20
Phthalylsulphathiazole†	0·5	6/20	6/20	0·5 0·25	1/20 0/10	1/20 0/10	7/10	0/10	1/20	10/20

* Numerator—number of chicks dead.
Denominator—number used.

†Results of two tests added together.
**Results of three tests added together.

‡Blood passed in faeces on the 5th to 7th day following infection.

when one day old from a single commercial hatchery and isolated as far as possible from the risk of extraneous coccidial infections, were inoculated orally with approximately 200,000 sporulated oöcysts of *Eimeria tenella*. Medication commenced 24 hours before or at various times up to 4 days after infection and in every case was terminated on the 7th day after infection. The strain of *E. tenella* employed, the method of housing the chicks, and the preparation of medicated proprietary food using 10 per cent or less w/w premixes of drug in plain wheat flour were similar to those already described (Ball, 1959). The activity of the mixtures was assessed by comparing the mortality in the treated and untreated groups; the passage of blood in the droppings was also recorded as a symptom of the acute stage of the disease. In order to observe delayed deaths after medication was terminated, the chicks were removed to clean cages for the remainder of the test. All chicks were examined post-mortem to confirm that the appearance of the caeca was consistent with acute caecal coccidiosis. In each experiment an uninfected untreated group was included, and no deaths were recorded from these at any time.

The potentiating effect of pyrimethamine upon the activity of sulphaquinoxaline was investigated by giving the drugs both singly and together in various proportions, commencing 2 days after infection. The concentrations of pyrimethamine necessary to produce a 50 per cent survival in chicks simultaneously receiving sub-effective concentrations of sulphaquinoxaline were obtained from dose-response curves, and the ED_{50} of each drug and for combinations of the two were plotted on a graph following the method used by Kendall and Joyner (1956b).

RESULTS

Effect of Commencing Medication before Infection

Groups of 7-day old chicks were given access to food containing various sulphonamides alone and mixed with pyrimethamine from one day before until the 7th day after infection, when the survivors were returned to normal food for another six or seven days. The number of deaths due to caecal coccidiosis occurring by the 7th and the 13th day following infection are recorded in Table 1. Sulphanilamide, sulphadiazine, sulphapyridine, succinylsulphathiazole and phthalylsulphathiazole given alone showed little or no effect, and sulphaguanidine, sulphamerazine and sulphathiazole were more active at the concentrations used. By the 7th day after infection, these sulphonamides, with the exception of sulphapyridine and succinylsulphathiazole, each mixed with 0·005 per cent w/w pyrimethamine, showed a significant increase in activity ($P < 0.05$) compared with that of the same or higher concentration of the sulphonamide given alone. In each case when an active drug mixture was removed, delayed haemorrhage occurred which was accompanied by deaths except in one sulphaguanidine-, one sulphanilamide-, and the phthalylsulphathiazole-pyrimethamine group, so that the synergistic effect of the majority of the mixtures was less evident by the 13th day after infection, i.e. 6 days after the withdrawal of medication.

The daily mortality of chicks fed various concentrations of sulphadimidine and sulphaquinoxaline mixed with pyrimethamine in a comparative test is shown in Table 2. All mixtures showed activity. In some groups haemorrhage and deaths occurred while the chicks were receiving the drug mixtures, whereas in other groups the mortality followed withdrawal of the drugs.

TABLE 2

EFFECT OF SULPHADIMIDINE OR SULPHAQUINOXALINE FED ALONE AND MIXED WITH PYRIMETHAMINE ON CAECAL COCCIDIOSIS IN CHICKS

Medication commenced one day before and terminated on the 7th day after infection

% drug in food			*Deaths from acute caecal coccidiosis*										
SD	*SQ*	*PYR*	*Days after infection*										*Total*
			5	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	
—	—	—	8	1									9/10
—	—	0·01	2		1					1			4/10
—	—	0·005	4	2	1								7/10
—	—	0·0025	5	2	1								8/10
0·1	—	—											0/10
0·05	—	—	3	3	2								8/10
0·025	—	—	5	2	1								8/10
0·025	—	0·005								2	1		3/9
0·025	—	0·0025			1								1/10
0·025	—	0·001		1	1								2/10
0·0125	—	0·005				1		2					3/10
—	0·0125	—	5										5/10
—	0·006	—	5	1	2								8/10
—	0·006	0·005								3			3/10
—	0·006	0·0025										1	1/10
—	0·006	0·001		1									1/9
—	0·003	0·005							1	1			2/10

SD = Sulphadimidine. *SQ* = Sulphaquinoxaline. *PYR* = Pyrimethamine

Effect of Sulphadimidine and Sulphaquinoxaline each with Pyrimethamine when Medication Commenced after Infection

The results of three experiments in which various treatments were started immediately or up to four days after inoculation are summarised in Table 3. All combinations possessed some therapeutic activity provided treatment was commenced before the fourth day after infection. However, when mixtures were given within 2 days of infection, deaths from the acute disease often followed the return to unmedicated food, but two associations (0·005 per cent pyrimethamine with 0·05 per cent sulphadimidine or 0·0125 per cent sulphaquinoxaline) fed for 4 days starting 3 days after infection gave good control with no delayed clinical symptoms.

TABLE 3

EFFECT OF SULPHADIMIDINE OR SULPHAQUINOXALINE MIXED WITH PYRIMETHAMINE ON CAECAL COCCIDIOSIS OF CHICKS WHEN GIVEN AFTER INFECTION

All treatments terminated on the 7th day after infection

% drug in food			*Time medication started after infection (days)*	*Deaths from acute caecal coccidiosis*										
				Days after infection										*Total*
SD	*SQ*	*PYR*		*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	
—	—	—	(Control)	19	8	2								29/31
—	—	0·005	2	11	6	2								19/24
0·2	—	—	2											0/10
0·1	—	—	2		4									4/10
0·05	—	—	2	4	4									8/10
0·025	—	0·005	Immediately						1	2				3/10
			1							4	1			5/10
			2							2				2/10
			3			2								2/11
			4	4	3									7/11
0·05	—	0·005	2		1						1			2/10
			3											0/11
			4	3	1									4/11
0·05	—	0·0025	2		1						2			3/10
0·05	—	0·0012	2	1	1									2/10
0·05	—	0·0006	2	1	3									4/10
—	0·0125	—	2	5	3									8/11
—	0·006	0·005	Immediately							3				3/10
			1						1	2		2		5/10
			2								1			1/10
			3		1									1/11
			4	7										7/11
—	0·0125	0·005	3											0/11
			4	7	2									9/11

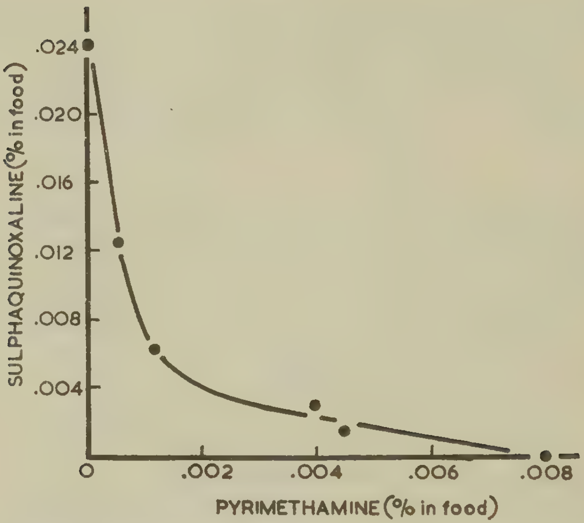
Abbreviations as in Table 2

The ED_{50} figures, as percentage drug in the food, for sulphaquinoxaline and pyrimethamine alone and in combination were plotted (Fig. 1) and produced a similar curve to that recorded by Kendall and Joyner (1956b) when demonstrating the potentiation of the action of sulphadimidine by pyrimethamine. Figure 1 therefore confirms that the action of sulphaquinoxaline was potentiated by pyrimethamine.

DISCUSSION

The experiments using pyrimethamine mixed with sub-effective concentrations of the sulphonamides, sulphadimidine, sulphaquinoxaline, sulphanilamide, sulphamerazine and sulphaguanidine

Fig. 1.



ED_{50}'s of sulphaquinoxaline and pyrimethamine administered both singly and together. (ED_{50} of sulphaquinoxaline alone was 0·024 per cent and of pyrimethamine was 0·008 per cent).

confirm in general the observation of other workers, referred to above, by showing that medication for 8 days, starting one day before the ingestion of *E. tenella* oöcysts, reduced deaths from severe caecal coccidiosis when the results were taken on the seventh day after infection, i.e., the last day of medication. The anticoccidial activity of sulphadiazine, sulphathiazole and phthalylsulphathiazole was also increased by the addition of pyrimethamine. However, three to five days after the termination of treatments haemorrhage invariably occurred, often accompanied by deaths from the acute disease, suggesting that these mixtures caused a temporary inhibition of the earlier endogenous stages of the coccidium with subsequent development after the withdrawal of the drugs. This delayed development was first noted by Joyner and Kendall (1955) following treatment with certain sulphadimidine-pyrimethamine mixtures, and was later recorded by Marthedal and Velling (1957) with combinations of sulphaquinoxaline and pyrimethamine.

Our findings with a number of sulphonamide-pyrimethamine associations given before infection and with various sulphaquinoxaline- and sulphadimidine-pyrimethamine mixtures started up to two days after infection indicate that a single course of treatment might not satisfactorily control a severe outbreak of caecal coccidiosis. Individual chicks of a flock may be harbouring parasites at different stages of development when medication is started and consequently treatment might only delay development of the early stages. On the other hand, the results given in Table 3 support the conclusion of Joyner and Kendall (1955) that certain mixtures have a selective effect on the later stages in the life cycle of the parasite. Therefore, medication should be limited to this period of development and the established intermittent treatment schedule may provide better control than a single course of treatment.

In general, the observations of Horton-Smith and Boyland (1946), Waletzky and Hughes (1946) and Schlenker and Simmons (1950) show that poorly absorbed sulphonamides have little or no anticoccidial activity in chicks and that the effect of the active ones parallel their blood concentrations. There is a close correlation between the relative anticoccidial efficiencies of eight well-known sulphonamides as set out by Horton-Smith (1957) and the relative blood concentrations in chicks of seven of them obtained by Kiser, Waters, Wirshup and Bottorff (1948), but as pointed out by Horton-Smith and Boyland (1946) absorption is not the only factor to be considered when comparing the activities of these compounds because although sulphapyridine is absorbed, it is inactive. It appears that the intestinal concentrations of sulphonamides may not be important in caecal coccidiosis as illustrated by Waletzky and Hughes (1946), who found that food concentrations of sulphaguanidine and sulphadiazine which produce comparable anticoccidial activity and blood concentrations produced different concentrations of drug in the caecal contents. The results with the poorly absorbed succinylsulphathiazole and phthalylsulphathiazole reported here support their findings.

The work of others, together with our results, provides a comparison of the activities of various mixtures based upon concentrations of drugs in the food, but there should be a closer quantitative relationship between activity and blood concentrations. There is little information on the absorption and excretion of sulphonamides and pyrimethamine given simultaneously to chicks, but studies of an exploratory nature in these laboratories indicate that approximately the same maximum blood concentration of sulphonamide is reached in chicks after the oral administration of equal amounts of sulphaquinoxaline alone or with the same quantity of pyrimethamine. Whether the rate of absorption of these drugs in mixture varies in individual chicks of different ages or whether there is a linear relationship between blood and tissue concentrations has not been ascertained.

CONCLUSIONS

Mixtures of pyrimethamine and the sulphonamides, sulphadiazine, sulphadimidine, sulphaguanidine, sulphamerazine, sulphanilamide, sulphaquinoxaline, sulphathiazole and phthalylsulphathiazole showed activity against severe experimental *E. tenella* infections in chicks when medication started one day before infection and continued for 8 days. These mixtures tended to cause only a temporary inhibition in the development of the parasite, and clinical symptoms of caecal coccidiosis often accompanied by deaths occurred after withdrawal of medication.

By comparison, treatment with 0·05 per cent w/w pyrimethamine plus 0·05 per cent w/w sulphadimidine or 0·0125 per cent w/w sulphaquinoxaline gave complete protection when given from the 3rd to the 7th day following infection.

The action of sulphaquinoxaline was potentiated by pyrimethamine.

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THERAPEUTIC EFFECT OF PYRIDINE-2-ALDOXIME METHIODIDE IN PARATHION POISONED MAMMALS

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INTRODUCTION

After successful experiments in vitro, where hydroxamic acids and oximes had been shown to reactivate cholinesterase inhibited by certain anticholinesterases as well as to react with certain inhibitors (Childs, Davies, Green and Rutland, 1955; Wilson, Ginsberg and Meislich, 1955), many experiments were performed in vivo in order to examine their therapeutic value in organophosphate poisoning.

It soon became evident that there was not always a close correlation between in vitro results and effects in vivo (Askew, 1956; Hobbiger, 1957). Pyridine-2-aldoxime methiodide (PAM) alone or in conjunction with atropine was a successful antidote in experimental poisoning with almost all the more common organophosphate compounds (for references see: Davies and Green, 1959) as well as in accidental Parathion (diethyl-*p*-nitrophenyl thiophosphate) poisoning in man (Namba and Hiraki, 1958; Karlog, Nimb and Poulsen, 1958). Davies and Green (1959) have recently discussed the rationale and development of newer antidotes to organophosphates and possible lines of future investigations. In the same article the authors have given a brief account of the range of compounds for which PAM has proved to be a successful antidote.

The study of the action of three oximes (PAM, diacetyl monoxime and mono-isonitrosoacetone) in small laboratory animals poisoned with Parathion showed that PAM was the only one with a therapeutic effect (Reiner, Svetlicic and Vandekar, 1957; Svetlicic and Vandekar, 1958). Further evaluation of the therapeutic action of PAM has been continued on larger animals.

This article summarizes our results on the effect of PAM in mice, rats, and rabbits poisoned with Parathion, together with some observations in dogs and horses, which are given in more detail. In all animals PAM showed a therapeutic effect when given after intravenous injection of Parathion. In dogs and horses the activities of cholinesterases in blood were estimated at short intervals, and the development and disappearance of symptoms roughly corresponded to the inhibition and reactivation of erythrocyte cholinesterase. At the same time it was found that the inhibitor persisted in the body longer than a single dose of PAM.

MATERIALS AND METHODS

Chemicals. Purified samples of Parathion were kindly supplied by Dr. G. Schrader, Farbenfabriken Bayer, A. G., Leverkusen, Germany.

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Two samples of PAM were used: the first, used in experiments on mice, rats, and rabbits, was a gift from the Chemical Defence Experimental Establishment, Porton, Wilts., England; the second, used in experiments on dogs and horses, was supplied by Dr. F. Raschig, GMBH, Chemische Fabrik; Ludwigshafen am Rhein, Germany*. Atropine sulphate (1 per cent. solution) was obtained from Vetserum Zavod, Zagreb, Yugoslavia. All chemicals were kept at 4°C, and dilutions were prepared immediately before use. Parathion was injected either as an emulsion in saline using Empilan as emulsifier (mice and rats) or as a solution in propylene glycol (rabbits, dogs and horses). PAM was given dissolved in saline in concentrations up to 33·3 mg./ml.

Animals. Male and female albino mice (20 to 25 g.) from the laboratory stock, male rats (150 to 200 g.) from the same stock, male chinchilla rabbits (Belgian hare) (2000 to 3000 g.) obtained from a local breeder, male and female crossbred dogs (5 to 18 kg.) obtained from a local knacker's premises, and male and female standard-bred horses aged 12 to 15 years (400-450 kg.) were used in the experiments. Mice and rats were maintained on the standard diet obtained from Vetserum Zavod, Zagreb. All the animals supplied were in good condition and were kept under observation at least two weeks before use.

Evaluation of the action of PAM. Acute toxicity tests were carried out on mice, rats and rabbits given Parathion intravenously. Similar tests were performed simultaneously on animals which received immediately after intravenous Parathion injection a single dose of PAM intraperitoneally (mice and rats) or intravenously (rabbits). 10 mice, 6 rats and 4 rabbits were used at each dose level. The dose levels were related by a common factor, i.e. 1·26 for mice and rats and 1·4 for rabbits. The LD50 and 95 per cent. confidence limits, given in brackets, were calculated by the method of Thompson (1947), and in instances where K was 3, tables for median-effective dose given by Weil (1952) were used.

Experiments on dogs and horses were carried out in order to follow the clinical symptoms and to estimate the activity of blood cholinesterases. The number of animals was not large enough to allow the calculation of LD50 by the above mentioned method. The animals were kept under observation until all symptoms disappeared, but never less than 48 hours.

Estimation of cholinesterase activity. At selected times blood (8 to 10 ml.) was collected from the vena saphena (dogs) or vena jugularis (horses) into 0·3 ml. of 30 per cent. sodium citrate and kept on ice. Ice cold buffer was used for washing erythrocytes. Estimations of cholinesterase activity of erythrocytes and plasma were carried out in the Warburg apparatus at 37°C, using acetylcholine chloride as substrate with final concentration of 0.0138 M (Aldridge, 1950). In all animals repeated estimations of normal enzyme activity were carried out (3 to 5 in dogs, and 5 to 8 in horses). The cholinesterase activities in poisoned animals are expressed as the percentage of the mean value of normal activities obtained in each animal.

RESULTS

Mice.

Intraperitoneal injection of PAM (150 mg./kg.) raised the intravenous LD50 of Parathion 2·5 to 3 times. Some of the

*This sample was a gift from Dr. A. Gladuli, Farmitalia S.A., Milano.

TABLE 1

EFFECT OF PAM ON THE INTRAVENOUS LD50 OF PARATHION IN MICE

PAM (150 mg./kg.) was given intraperitoneally immediately after i.v. injection of Parathion

LD50 (mg./kg.)	*Male*		*Female*	
	Parathion	*Parathion + PAM*	*Parathion*	*Parathion + PAM*
After 24 hours	11·1 (9·32–12·7)	40·6 (34·4–47·9)	15·1 (13·4–17·0)	38·0 (35·2–41·1)
After 48 hours	10·4 (9·17–11·8)	31·6 (28·7–34·8)	14·4 (12·7–16·3)	33·2 (30·2–36·4)

treated animals survived the 24-hour period, but died later within 48 hours, the 24-hour LD50 values showing consequently a somewhat larger difference between untreated and treated animals (Table 1). The single injection of PAM resulted not only in reducing the number of deaths, but also in abolishing certain symptoms such as salivation and fasciculations at lower doses of Parathion and prolonging the survival time at higher doses. More characteristic symptoms observed in female mice at some of injected doses are given in Table 2.

TABLE 2

EFFECTIVENESS OF PAM (150 MG./KG., I.P.) GIVEN IMMEDIATELY AFTER INTRAVENOUS PARATHION INJECTION IN FEMALE MICE

Only dosages with most characteristic symptoms are given

PAM (mg./kg.)	*Parathion (mg./kg.)*	*Symptoms observed* *Time in minutes after injection is given in brackets*	*Fatality rate*
0	6·0	No symptoms (20), less active (30), normal (60)	0/10
0	12·0	Slight tremor (6-8), generalized pronounced fasciculations, salivation, very pronounced tremor, progressive dyspnoea and convulsions (10), deaths (10-30), or slow recovery with no symptoms after 5-6 hours	4/10
0	24·0	Very pronounced salivation, tremor, and dyspnoea, severe convulsions (3), deaths (6-14)	10/10
150	24·0	Slight tremor (5-9), very pronounced tremor, and dyspnoea, moderate convulsions (15), progressive recovery, no convulsions, moderate tremor in less and less frequent attacks (30), slight tremor (120), no signs of poisoning next day	0/10
150	48·0	Very pronounced tremor, salivation, dyspnoea, and convulsions (4-5), very pronounced weakness, moderate tremor in attacks (60), very pronounced dyspnoea, prostration, and death in 12-24 hours	10/10

Rats

The effect of PAM (100 mg./kg. i.p.) on male rats given Parathion intravenously was tested on two occasions, using two different samples of Parathion (Table 3). The difference in the intensity of symptoms and the fatality rate between untreated and treated animals was not very marked, but when comparing the survival time at the given doses a certain therapeutic effect of PAM becomes apparent (Table 4).

TABLE 3

EFFECT OF PAM ON THE INTRAVENOUS LD50 OF PARATHION IN MALE RATS

PAM (100 mg./kg.) was given intraperitoneally immediately after i.v. injection of Parathion

LD50 (mg./kg.)	*Parathion*	*Parathion + PAM*
Sample 1	7·57 (7·12–8·04)	13·1 (9·56–17·9)
Sample 2	10·1 (8·20–12·4)	15·0 (13·3–16·8)

TABLE 4

EFFECTIVENESS OF PAM (100 MG./KG., I.P.) GIVEN IMMEDIATELY AFTER INTRAVENOUS PARATHION INJECTION IN MALE RATS

PAM (mg./kg.)	*Parathion (mg./kg.)*	*Cholinergic symptoms*	*Time of death (min.)*	*Fatality rate*
0	6·0	none	—	0/6
0	8·5	very pronounced	14–55	4/6
0	12·0	very pronounced	9–25	2/6
0	17·0	very pronounced	5–11	6/6
0	24·0	extremely severe	3–7	6/6
100	8·5	slight to moderate	—	0/6
100	12·0	moderate	—	0/6
100	17·0	very pronounced	28–180	5/6
100	24·0	severe	10-34	6/6

Rabbits.

The therapeutic effect of PAM was demonstrated also in rabbits. The LD50 for Parathion given intravenously was estimated as 5·95 mg./kg. with 95 per cent. confidence limits from 4·09 to 8.64 mg./kg. In treated animals which immediately after Parathion injection received a single dose of PAM (100 mg./kg. i.v.), the LD50 was estimated as 22·0 mg./kg. with 95 per cent. confidence limits from 16·5 to 28·0 mg./kg. Intensity and duration of symptoms and survival time as well as the fatality rate for given doses of Parathion in untreated and treated animals are given in Table 5.

Dogs

Two groups of four animals were given Parathion only (3·0 and 6·0 mg./kg., i.v.) and the third was treated with PAM (50 mg./kg. i.p.) immediately after the intravenous injection of Parathion (6.0 mg./kg.). Each group consisted of two male and two female dogs. The clinical symptoms were recorded and the activity of erythrocyte cholinesterase was estimated at selected intervals.

One of four dogs injected with 3·0 mg./kg. of Parathion showed slightly pronounced salivation, tremor and dyspnoea which ceased within 1 hour. All the animals given 6.0 mg./kg. developed very pronounced salivation, generalized fasciculations, tremor, dyspnoea and convulsions, which eventually led to extreme prostration and death between one and a half and 23 hours. The animals given both Parathion and PAM developed slight salivation, pronounced tremor and hardly noticeable dyspnoea within 15 to 30 minutes, but these symptoms started to decline one hour after the injections. No fasciculations or convulsions were observed, and all four animals survived. Whereas the activity of erythrocyte cholinesterase in untreated animals was substantially reduced, no marked inhibition was found in treated animals within the first three hours (Fig. 1), but after 24 and 48 hours the activity was reduced to about 50 to 65 per cent. This delayed inhibition was not followed by any clinical symptoms.

Horses

Two male horses (Nos. 1 and 2) were injected with Parathion intravenously, 1·5 and 3·0 mg./kg., respectively. Both animals showed only minimal symptoms, i.e. slight salivation, exaggerated peristalsis, slight watery nasal discharge, moderately increased bronchial secretion with ronchi, cough and slight dyspnoea. These symptoms started half an hour after injection and disappeared within three hours. The degree of inhibition of erythrocyte and plasma cholinesterase was similar in both animals, the activities approaching 40 per cent. for erythrocyte and 10 per cent. for plasma. Although next day the animals appeared normal and cholinesterase activities showed a tendency to increase, PAM (20 mg./kg.) was injected intravenously and the reactivation of enzymes was estimated

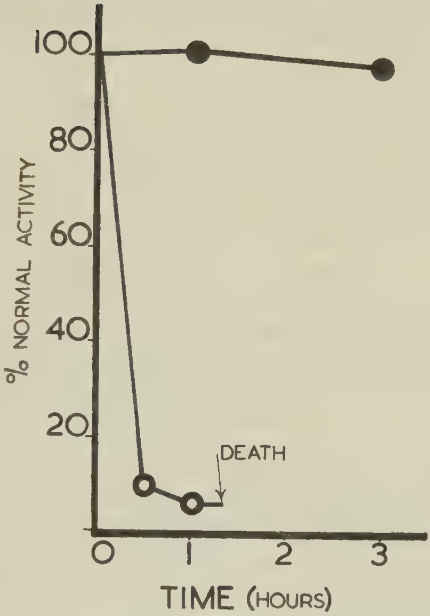
TABLE 5

EFFECTIVENESS OF PAM (100 MG./KG.) GIVEN IMMEDIATELY AFTER INTRAVENOUS PARATHION INJECTION IN MALE RABBITS

Intensity of symptoms: (—) no; (+) slight; (+ +) moderate; (+ + +) very pronounced

	Parathion								*Parathion + PAM*							
Parathion (mg./kg.	*Salivation*	*Fasciculations*	*Tremor*	*Dyspnoea*	*Convulsions*	*Duration of symptoms (hrs.)*	*Time of death*	*Fatality rate*	*Salivation*	*Fasciculations*	*Tremor*	*Dyspnoea*	*Convulsions*	*Duration of symptoms (hrs.)*	*Time of death*	*Fatality rate*
2·5	—	++	+	++	—	3–5	—	0/4								
3·5	++	++	++	++	—	6–7	3 hrs	1/4								
5·0	+++	+++	+++	++	+	6	1-2 hrs	2/4								
7·0	+++	+++	+++	+++	++	24	1 hr	2/4								
10·0	+++	+++	+++	+++	+++	24	15-45′	3/4	—	—	+	+	—	2	—	0/4
14·1	+++	+++	+++	+++	+++	—	14-21′	4/4	+—	—	+	+	—	2-3	—	0/4
20·0									+	—	++	+++	++	24	8-10′	2/4
28·2									+	+—	++	+++	+++	24	6′	3/4
40·0									—	—	+++	+++	+++	—	2′	4/4

FIG. 1



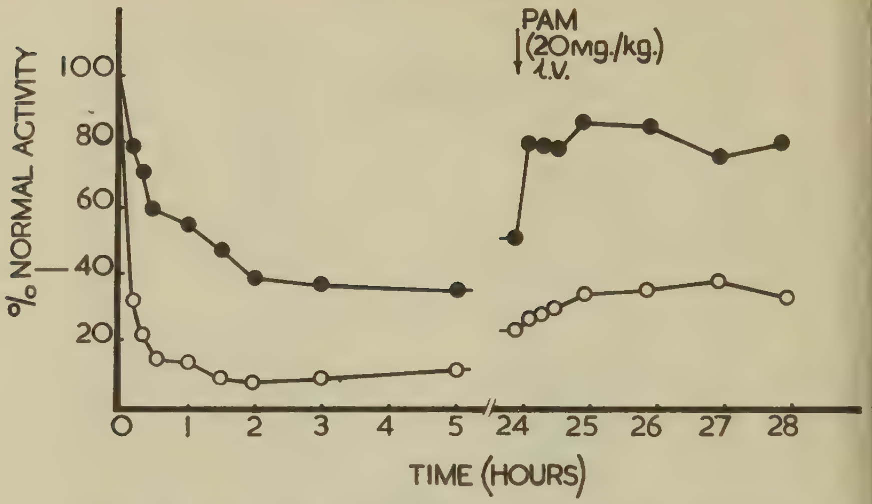
Two female dogs were injected with Parathion (6·0 mg./kg.) intravenously at "zero time". Erythrocyte cholinesterase activity is shown in blood samples taken from the dog which received no treatment (o), and from the animal which was given PAM (50 mg./kg. i.p.) immediately after Parathion (●).

(Fig. 2). Only the erythrocyte cholinesterase was markedly reactivated.

A female horse (No. 3) was given 6·0 mg./kg. of Parathion intravenously. At the moment when the animal developed extremely severe symptoms and prostration intravenous injection of PAM (20 mg./kg.) was given, the same dose being repeated after 1 hour. PAM showed a pronounced effect, abolishing the symptoms rapidly. Erythrocyte cholinesterase was markedly reactivated, whereas plasma cholinesterase activity was changed only slightly, the values approaching 20 per cent. The clinical course of poisoning is given in Table 6.

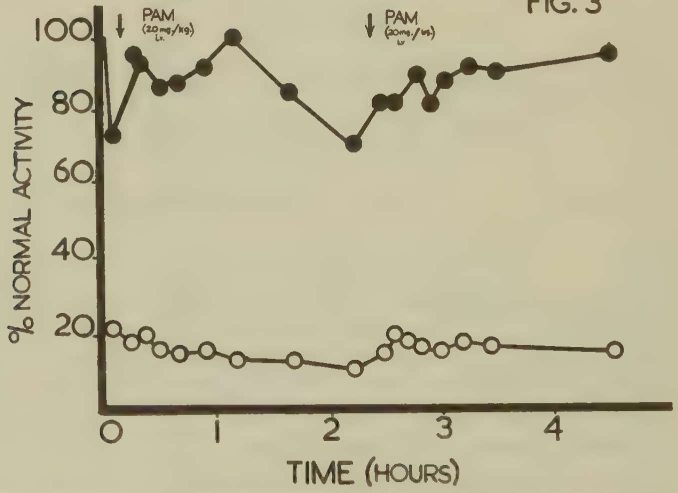
In order to test the efficiency of PAM in controlling the symptoms when given before they appeared, a dose of 20 mg./kg. i.v. was injected into mare No. 4 10 minutes after Parathion (6·0 mg./kg. i.v.). Only slight symptoms, such as occasional muscular twitchings in the neck and the foreleg muscles, very slight dyspnoea and minute watery nasal discharge, were observed. The animal was quiet and accepted the food offered. Two hours later the injection of the same

FIG. 2.



A male horse (No. 2) was injected with Parathion (3·0 mg./kg.) intravenously at "zero time". Next day PAM (20 mg./kg.) was given intravenously. The activity of erythrocyte (●) and plasma (o) cholinesterase in blood samples taken at given intervals is shown.

FIG. 3



A female horse (No. 4) was injected with 6·0 mg./kg. Parathion intravenously at "zero time". 10 and 130 minutes later PAM (20 mg./kg.) was given intravenously. The activity of erythrocyte (●) and plasma (o) cholinesterase in blood samples taken at given intervals is shown.

dose of PAM was repeated, after which no more symptoms of poisoning were observed. The third dose of PAM was given next day. The cholinesterase activity was estimated at frequent intervals during the first 4 hours (Fig. 3) and at intervals of a few days up to the 16th day. The erythrocyte cholinesterase was not inhibited below 75 per cent. of normal activity during the first 4 hours (Fig.3). During the next few days the activity slowly decreased and on the fifth day reached 40 per cent. Subsequently there was a very slow

TABLE 6

ABRIDGED CLINICAL PROTOCOL OF MARE (No. 3) POISONED BY PARATHION AND TREATED WITH PAM

Time (min.)	*Symptoms observed*	*Erythrocyte cholinesterase activity* (%)
0	Intravenous injection of Parathion (6·0 mg./kg.)	
2	Moderate muscular twitchings, trembling and cyanosis	
5	Recovery, no symptoms	90
10	Moderate salivation and rhinorrhea	45
20	Restlessness, slight dyspnoea, hyperexcitability	17
25	Tremor of the head, moderate dyspnoea, pronounced salivation	
30	Generalized fasciculations and tremor, severe dyspnoea, pronounced salivation, ataxia and convulsions	
35	Unconsciousness, the skin bathed in cold perspiration, aimless movements, staggering, more and more frequent convulsions (tonic and clonic), dacryorrhea	
65	Extremely severe dyspnoea with wheezing expiration and ronchi, excessive salivation and watery nasal discharge, very pronounced tremor and fasciculations, prostration	15
70	Intravenous injection of PAM (20 mg./kg.)	
75	Convulsions gradually disappear; fasciculations and tremor present	
80	No convulsions, still unconscious, respiration gradually getting normal	
85	No dyspnoea, moderate tremor and fasciculations	115
90	Corneal reflex and consciousness re-established	
130	No salivation, trying to get on its feet	98
140	Intravenous injection of PAM (20 mg./kg.)	
145	No tremor, slight fasciculations, showing appetite for the food offered	
170	Dry skin	123
200	Up on its feet	115
260	No symptoms of poisoning but slight isolated fasciculi, eating	
320	Somnolent; no other symptoms	93

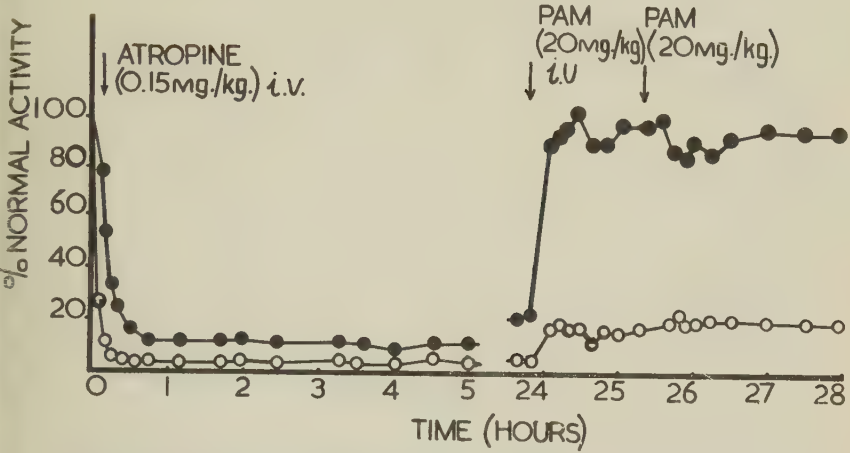
spontaneous recovery. No symptoms followed this delayed inhibition. The plasma cholinesterase activity fell in the first 10 minutes as low as 10 to 20 per cent., showing only a slight rise after PAM injections. The activity returned slowly and reached not more than 25 per cent. in 16 days.

A mare (No. 5) was given 6·0 mg./kg. of Parathion intravenously and PAM was given 24 hours later. In the interval the animal was treated only with atropine, the first dose (0·15 mg./kg. i.v.) being given 5 minutes after the Parathion injection. No symptoms attributable to anticholinesterase poisoning were observed during the first hour and the animal showed only signs of mild atropinization, such as dilated pupils, dry mouth and tachycardia (88 per minute). During the second hour fasciculations started in the masseters and hindleg muscles. After two hours, generalized fasciculations were present together with other cholinergic symptoms: salivation, sweating, myosis, slight tremor and dyspnoea. The intensity of symptoms increased from hour to hour and after 7 and a half hours the animal became prostrate, showing extremely pronounced symptoms as mentioned above, and convulsions. Profuse sweating was present. At this moment an intravenous injection of atropine was repeated in the same dose. Recovery was more than rapid; salivation, tremor and dyspnoea ceased in 5 minutes and within 45 minutes the skin became completely dry. Fasciculations decreased gradually, but persisted for 2 hours. Next day mild symptoms of poisoning were present again—slight salivation and tremor, anorexia and slight depression. At this time PAM (20 mg./kg.) was given intravenously and repeated after one and a half hours. After PAM administrations the animal did not show any symptoms except a slight depression and persistent constipation which was cured after repeated deep enemas.

Figure 4 shows the erythrocyte and plasma cholinesterase activities, which decreased rapidly after Parathion injection to 15 and 5 per cent. respectively and did not change during the next 5 hours. The same figure shows the reactivation produced by PAM given 24 hours after Parathion.

A mare (No. 6) was given 9·0 mg./kg. of Parathion intravenously. Immediately after injection there was pronounced dyspnoea, muscular twitchings, restlessness and tremor, but these ceased within 2 to 3 minutes. Similar transient symptoms, only less marked, were also observed in the horses given 6·0 mg./kg. of Parathion. (Table 6). After an interval of 10 minutes, characterized by no symptoms, the cholinergic symptoms gradually developed. 20 minutes after the Parathion injection pronounced salivation and muscular twitching were present and after 30 minutes, very pronounced generalized fasciculations, extreme salivation, rhinorrhea, continuous moderate tremor as well as profuse sweating, firstly in the neck and chest region, and then all over the body, were observed. After 40 minutes very pronounced dyspnoea, ataxia and anxiety were present, the

FIG. 4

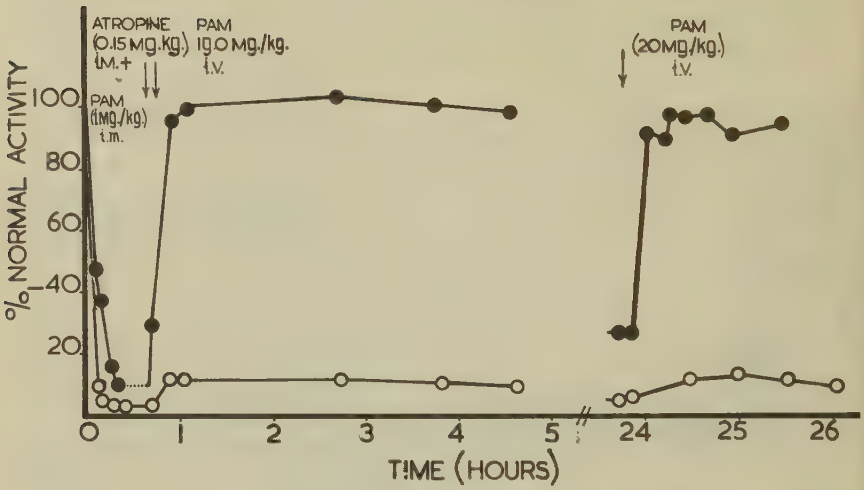


A female horse (No. 5) was injected with 6·0 mg./kg. Parathion intravenously at "zero time". 5 minutes and 7·5 hours later atropine (0·15 mg./kg.) was given intravenously. Next day 2 intravenous injections of PAM (20 mg/kg.) were given. The activity of erythrocyte (●) and plasma (o) cholinesterase in blood samples taken at given intervals is shown.

animal became unconscious and in one of the severe attacks of tremor fell down and lay exhausted on its side. Prolonged, very loud wheezing expiration (laryngeal spasm) and moderate cyanosis were present. At this moment 0·15 mg./kg. of atropine together with 1·0 mg./kg. of PAM were given intramuscularly, and 8 minutes later, when the animal became more tranquil, 19 mg./kg. of PAM were administered intravenously. A rapid decrease in the symptoms was observed and within 10 minutes the animal tried to get on its feet. Respiration became gradually normal and salivation, sweating, fasciculations and tremor ceased. Pronounced mydriasis appeared and the animal got on its feet 30 minutes after administration of antidotes. Half an hour later the animal was behaving normally, eating and drinking the food offered. Next day the animal again showed symptoms of poisoning in the form of moderate salivation and fasciculations, and slight dyspnoea. These symptoms disappeared soon after PAM injection (20 mg./kg. i.v.), and recovery was permanent.

The erythrocyte and plasma cholinesterase activities found during 26 hours of the experiment are given in Fig. 5. Even the small amount of PAM (1·0 mg./kg.) given intramuscularly raised the erythrocyte cholinesterase activity up to 30 per cent. and the remaining amount of PAM (19 mg./kg.) given intravenously reactivated the enzyme up to 90 to 100 per cent. These values did not change within first 4 hours, but the next day activity was found to

FIG.5.



A female horse (No. 6) was injected with 9·0 mg.kg. Parathion intravenously at "zero time". 40 minutes later atropine (0·15 mg./kg.) and PAM (1·0 mg./kg.) were injected intramuscularly, intravenous injection of PAM (19 mg./kg.) followed 8 minutes later. Next day PAM (20 mg./kg.) was given intravenously. The activity of erythrocyte (●) and plasma (*o*) cholinesterase in blood samples taken at given intervals is shown.

be under 30 per cent. The injection of the second dose of PAM again produced a rapid and marked reactivation of erythrocyte cholinesterase. On the third and fifth days respectively, the activity was between 45 to 50 per cent, but no symptoms were present. The inhibited plasma cholinesterase did not exceed 10 per cent. during this period.

DISCUSSION

Pure Parathion is a very poor inhibitor of cholinesterase and is converted in vivo to Paraoxon which is the real lethal agent (Diggle and Gage, 1951). In practical cases of Parathion poisoning the toxic action of highly active impurities, such as Parathion isomers and Paraoxon, will be included (Aldridge, and Barnes, 1952). Our samples of Parathion, although "chemically pure", contained traces of active inhibitors undetectable by chemical means. In vitro estimation of the anticholinesterase activities showed that the amount of active impurities did not exceed 0·1 per cent. The rapid onset of cholinergic symptoms observed in mice, rats and rabbits after higher doses of Parathion must have been due, to a great extent, to the presence of the active inhibitors in the given sample. Nevertheless, the amount of active impurities was small enough to allow PAM to exhibit a pronounced therapeutic effect even when given after intravenous injection of Parathion.

The therapeutic effect of PAM in animals poisoned with Parathion depends not only upon the time and the route of antidote administration, but also on the rate of its elimination, as well as on the rate of conversion of Parathion to Paraoxon in the given species. These factors are mainly responsible for the differences in the therapeutic effect of PAM observed in mice, rats and rabbits. Slow conversion of Parathion to Paraoxon may diminish the therapeutic effect of a single dose of antidote if the latter is eliminated rapidly. The differences in 24 and 48-hours LD50 in mice treated with PAM indicate that PAM (150 mg./kg. i.p.) had been eliminated before the whole amount of injected Parathion was metabolised. These results agree with the findings obtained in mice poisoned with a highly purified sample of Parathion (Wilson and Sondheimer, 1957). Further evidence that PAM is eliminated long before Parathion is converted to Paraoxon is given by the delayed inhibition of erythrocyte cholinesterase observed in dogs and horses 24 hours after injection of PAM.

PAM produced its therapeutic effect not only by counteracting the nicotinic signs, but also, at lower doses of Parathion, by abolishing the muscarinic and central nervous system symptoms. Intravenous injection of PAM (20 mg./kg.) exerted a marked effect in restoring consciousness within 20 minutes in a horse poisoned with 6·0 mg./kg. of Parathion. A similar effect of PAM was noted in a case of suicidal Parathion poisoning (Karlog *et al.*, 1958).

In dogs, blood samples were taken at intervals which were not frequent enough for an adequate comparison of the enzyme inhibition with the clinical symptoms. In the untreated animals, given Parathion 6·0 mg/kg. i.v., deaths occurred after the erythrocyte cholinesterase activity was reduced below 10 per cent. In the animals treated with PAM there was no marked inhibition of the erythrocyte cholinesterase activity although moderate symptoms were observed. The frequent collection of blood samples in horses allowed a close comparison of the enzyme inhibition with clinical symptoms. As the brief transient symptoms observed within the first few minutes after intravenous injection of Parathion (6·0 or 9·0 mg./kg.) ceased before the blood cholinesterases were markedly inhibited, their appearance was very probably due to the direct, non-specific central action of Parathion. The first cholinergic symptoms appeared after erythrocyte cholinesterase activity was reduced below 50 per cent., whereas for very pronounced symptoms the activity corresponded to values around 15 per cent. In all instances the plasma cholinesterase was inhibited faster and more markedly than the erythrocyte cholinesterase. After injection of PAM the erythrocyte cholinesterase was markedly reactivated: the extremely rapid reactivation of the inhibited erythrocyte cholinesterase was followed by a gradual disappearance of symptoms. The action of PAM on the inhibited plasma cholinesterase was very poor.

Although all blood samples were kept on ice before cholinesterase activity determination, an additional reactivation of the inhibited enzyme in vitro cannot be excluded (Hobbiger, 1957). Nevertheless the results of kinetic studies on the reactivation of the inhibited cholinesterase in vitro show that this reaction is very slow at temperatures near 0°C (Davies and Green, 1956). In horse No. 3 the erythrocyte cholinesterase activity was found repeatedly above the original values after injection of PAM. One explanation for these findings may be haemoconcentration, although the intensity of salivation and sweating did not appear to be greater than that observed in horse No. 6 receiving higher dose of Parathion.

PAM was shown to have the same efficiency in reactivating the inhibited erythrocyte cholinesterase if given 24 hours after Parathion, indicating that only a very small amount of diethylphosphorylated enzyme was converted to the unreactivable form. The delayed administration of PAM in Parathion poisoning is therefore justified and its successful antidotal action may be presumed.

Regarding the dosage of PAM, a marked reactivation of the inhibited erythrocyte cholinesterase was produced even with a dose as low as 1·0 mg./kg. given intramuscularly. However, as mentioned above, the treatment of Parathion poisoning differs from that of poisoning with other organophosphorus compounds because of the slow conversion of Parathion to Paraoxon. The experiment on horse No. 6 serves as a good example: in spite of full and rapid recovery after combined treatment with PAM and atropine and in spite of rapid reactivation of the inhibited erythrocyte cholinesterase, symptoms of poisoning developed again within 24 hours, and the inhibition of erythrocyte cholinesterase occurred once more. Thus large and repeated doses of PAM are necessary in order to restore to health animals poisoned with Parathion.

CONCLUSIONS

The therapeutic action of PAM was studied in mice, rats, rabbits, dogs and horses given Parathion intravenously. In mice, rats and rabbits LD50 values for untreated and treated animals were estimated. A single dose of PAM injected in mice (150 mg./kg. i.p.) or rabbits (50 mg./kg. i.v.) raised the intravenous LD50 of Parathion about 3 times. In rats the therapeutic effect of PAM (100 mg./kg. i.p.) was only slight.

Dogs and horses poisoned with Parathion, were given PAM 50 mg./kg. i.p. and 20 mg./kg. i.v. respectively. In addition to the observation of clinical symptoms the cholinesterase activity in blood was estimated. PAM reactivated the inhibited erythrocyte cholinesterase very markedly, whereas its action on the inhibited plasma cholinesterase was hardly noticeable. At lower doses of Parathion, PAM showed an ability to counteract the nicotinic, muscarinic and central nervous system signs of anticholinesterase poisoning. In spite of marked reactivation of the inhibited erythrocyte cholinesterase and complete disappearance of symptoms after a single dose of PAM next day, the erythrocyte cholinesterase was found to be inhibited again and in some instances cholinergic symptoms returned. As the conversion of Parathion to Paraoxon is slow, large and repeated doses of PAM should be given in order to obtain prolonged therapeutic effect.

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POST MORTEM GLYCOLYSIS IN NORMAL AND EXUDATIVE LONGISSIMUS DORSI MUSCLES OF THE PIG IN RELATION TO SO-CALLED WHITE MUSCLE DISEASE

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INTRODUCTION

In recent years reference has been made by Danish and French investigators to a dystrophic condition in the muscles of pigs, which is superficially characterized by a pale, exudative appearance of the flesh (Ludvigsen, 1954, 1955; Henry, Billon and Haouza, 1955; Henry, Romani and Joubert, 1958). A condition of this kind was apparently recorded in Germany 50 years ago (Wismer-Pedersen, 1959), although its aetiology was then unknown. In Denmark the dystrophy, "muskeldegeneration" disease, appears to be prevalent in pigs of the Landrace breed. A striking biochemical aspect is the low pH of the musculature in the immediate post mortem period, values of 5·4 being attained 30 mins. after death; the pH is about 6·4 at this time in "normal" Danish pigs (Ludvigsen, 1954, 1955). Ludvigsen indicated that the longissimus dorsi muscle was particularly susceptible to the condition. He regards it as one of the diseases of adaptation to which easily stressed individuals are subject (Selye, 1936, 1950). Wismer-Pedersen (1959) has also studied the exudative phenomenon in Danish Landrace pigs and, likewise, has associated it with a very low pH in the musculature soon after death.

The name "la myopathie exsudative dépigmentaire du porc", given to the dystrophy by the French investigators, its recognition as a disease of adaptation and the histological changes observed (Henry *et al.*, 1955, 1958) indicates that it is generally similar to "muskeldegeneration." On the other hand the exudative pork muscles examined by Henry *et al.* (1955) have a normal initial pH, as measured immediately post mortem; they have, however, a lower ultimate pH, ca. 5·2, in comparison with a so-called normal value of ca. 5·9. Both Ludvigsen and Henry *et al.* found marked histological changes in dystrophic muscle, but these were not observed by Wismer-Pedersen. Presumably there may be abnormal degrees of exudation in pig muscle which are not as severe as in "muskeldegeneration."

There has been a sporadic incidence of pale, exudative pig muscle in the United Kingdom. An opportunity has been taken of examining this material, of comparing its characteristics with those described by the French and Danish investigators and of considering the general implications of the condition. The longissimus dorsi muscle has been investigated principally, both in view of its major size and importance in the animal and because of its frequent involvement in the syndrome. It has also seemed particularly desirable to establish the meaning of "normal" glycolysis in the longissimus dorsi muscle of the pig, especially in relation to this process in the longissimus dorsi muscles of other domestic species.

METHODS

Sampling.

Samples of the muscles studied were taken from the following regions of the carcases (right hand side). Longissimus dorsi, region of 4th, 5th and 6th lumbar vertebrae (unless otherwise stated); psoas, portion of psoas major at level of 1st sacral vertebra; diaphragm, junction of pars sternalis with pars costalis; semimembranosus and rectus femoris, peripheral portions at a level approximately 2″ distal from head of femur. Except in glycolysis experiments samples were excised from the carcase 24 hours post-mortem.

Procedure in glycolysis experiments

Pigs were slaughtered at some distance from laboratory facilities. In order to standardize conditions as far as possible, investigation of biochemical events during post-mortem glycolysis in the muscle samples commenced at exactly one hour post-mortem. This will be regarded as the "initial" time in the present paper. Muscles were held in moist nitrogen at 37°C to maintain complete anaerobiosis, and thus ensure freedom from the complications of respiration, and to avoid the build-up of intermediary phosphate esters which occurs at room temperature. For the measurement of the extensibility changes associated with the onset of rigor mortis thin strips of muscle about 0·8 cm. wide and 3 cm. long were dissected from the samples. Changes in extensibility were followed in the apparatus of Bate-Smith and Bendall (1949) and were expressed as a percentage of the initial value. Portions of the sample adjacent to the strips were taken for the measurement of pH and for the preparation of phosphate-containing extracts.

Exudation

The loss of moisture from muscle samples suspended in moist nitrogen at 37°C was expressed as a percentage of the initial weight.

Chemical estimations

pH. Approximately 1 g. of muscle was macerated in 10 ml. of 0·01 M-sodium iodoacetate in a Marsh-Snow homogenizer (Marsh and Snow, 1950) and the pH measured by glass electrode.

Ultimate pH represented the value at 24 hours post mortem, when lactic acid production from glycogen had ceased: in this case maceration was carried out in distilled water.

Buffering power. This was determined as described previously (Lawrie, 1953a).

Glycogen. Determination of glycogen was carried out by the method of Good, Kramer and Somogyi (1933). "Initial" and "residual" glycogens refer respectively to the levels at 1 hour and 24 hours post mortem. In the latter case, samples were held under moist nitrogen at 37° for 5 hours, i.e. until glycolysis had virtually ceased, and then for a further 17 hours at 0°C.

Lactic acid. The method of Friedemann and Graeser (1933) was used, after treatment of extracts with copper lime to remove reducing compounds, and with minor modifications to the distillation apparatus.

Myoglobin. This was estimated as alkaline haematin (Lawrie, 1950).

Phosphate. The preparation of phosphate-containing muscle extracts and the estimation of total acid-soluble phosphorus (TSP), creatine phosphate phosphorus (CP/P) and adenosine triphosphate phosphorus (ATP/P) followed, generally, the methods used hitherto (Lawrie, 1953a); but, in determining ATP/P, hydrolysis with N-HCl at 100°C was carried out for 10 instead of 7 min. Values for ATP/P and CP/P were expressed as a percentage of TSP. In certain instances the method of Bailey and Marsh (1952) was used to correct for the presence of traces of hexose diphosphate and triose phosphates in the extracts.

RESULTS

Initial and Ultimate pH in the Normal Longissimus Dorsi Muscle

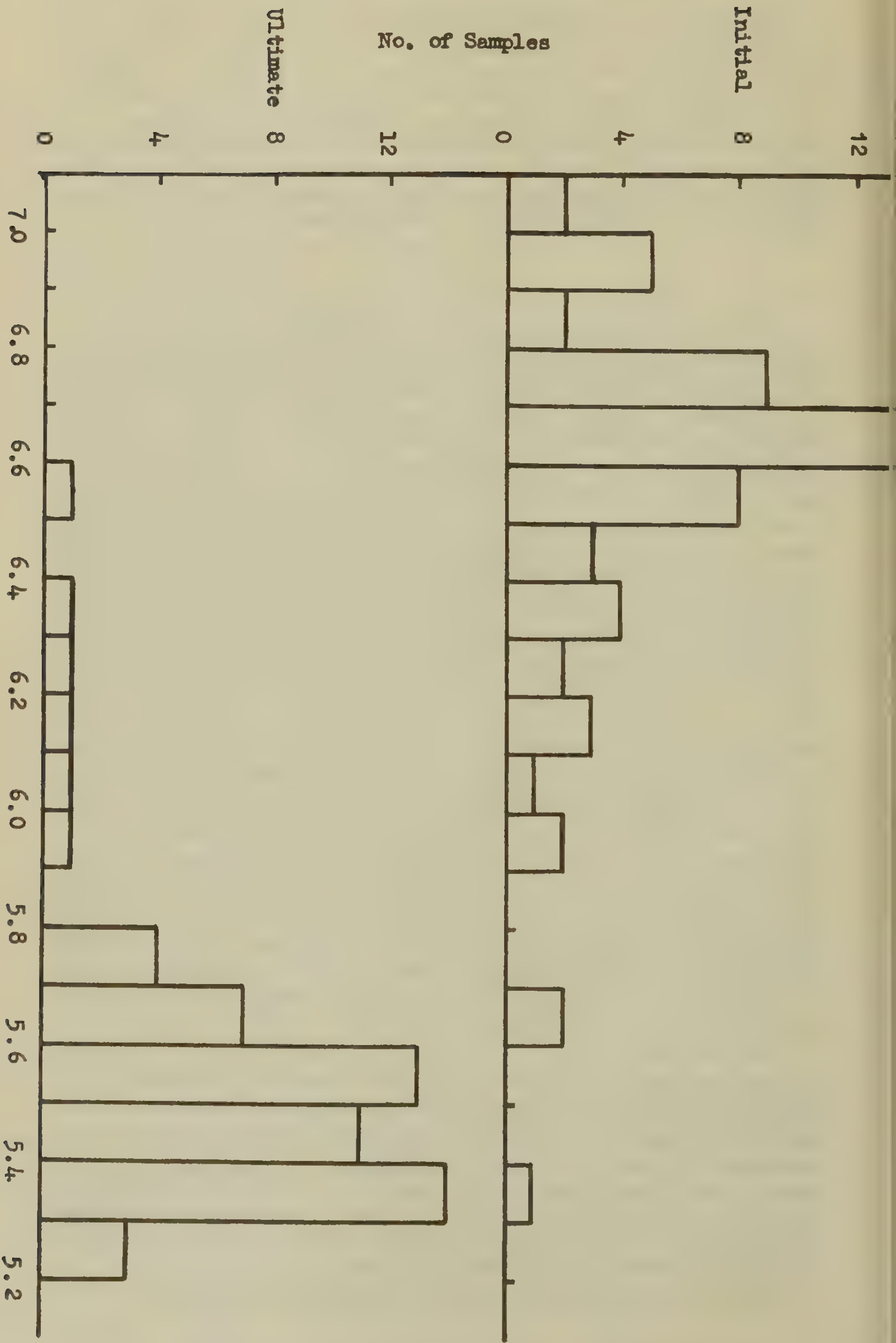
The mean values for the initial and ultimate pH of longissimus dorsi muscles from a series of 58 unselected pigs, predominantly of Large White x Middle White breeds, were 6·54 and 5·55 respectively. The latter agrees with the data of Callow (1937), and Callow, Ingram and Hawthorne, (1939), for pigs of this breed, but is distinctly lower than the mean ultimate pH of 5·77 quoted by German workers (Schön and Stosiek, 1958) and higher than the corresponding data (5·30) of Ludvigsen (1954, 1955) for Danish pigs of the Landrace breed. From Fig. 1, in which is represented the frequency distribution of the data, it will be seen that the values for initial pH are markedly more variable than those for ultimate pH. It could thus be assumed that there is more variation between individual pigs in the degree of struggling at death than in the degree of inanition or exhaustion ante-mortem. The former factor is the principal determinant of initial pH (Bate-Smith and Bendall, 1949); the latter factors are mainly responsible for controlling the level of glycogen available immediately before death for lactic acid production (Bernard, 1877; Hoet and Marks, 1926; Bate-Smith, 1936; Callow, 1936) and, hence, for the ultimate pH. It is also clear from Fig. 1 that about 1 in 3 of the initial pH values lie below 6·5 and approximately the same proportion of ultimate pH values lie above 5·6. For longissimus dorsi muscles from 30 control (untreated) steers only three pH values were found to lie below, and one value above, these respective limits (Howard and Lawrie, 1956, 1957). Pigs apparently tend to struggle more at death than oxen and to be more susceptible to ante-mortem factors which deplete muscle glycogen stores.

Despite this variability in pig longissimus dorsi, there are nevertheless definite maxima in the frequency of pH values (Fig. 1). The mean initial and ultimate pH, based on the measured values within the ranges 6·5 to 6·8 and 5·3 to 5·6, respectively, and thus excluding aberrant data arising from undue struggling at death or preslaughter stress, are ca. 6·7 and ca. 5·5: these values may be regarded as typical of the normal longissimus dorsi muscle of the pig.

Comparative Aspects of Post-mortem Glycolysis

The normal progress of post mortem glycolysis between pH 6·7

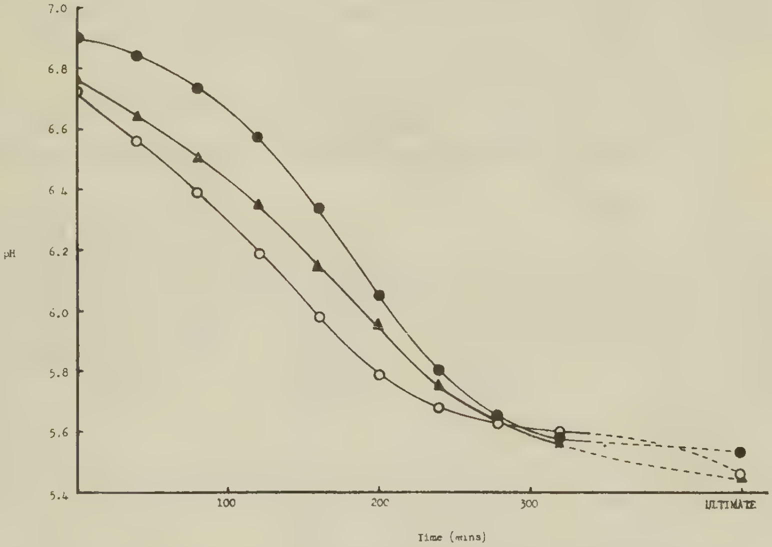
Fig. 1



Initial and ultimate pH in pig l. dorsi muscles. Frequency distribution of values from 58 unselected animals.

and pH 5·5, in moist nitrogen at 37°C, is shown as a mean pH/time curve in Fig. 2, together with corresponding curves for typical longissimus dorsi muscles of the ox and the horse. Although the initial pH in horse longissimus dorsi is distinctly higher than that in ox and pig, nevertheless, in all three species a pH of 5·6 is reached after about 300 min. It is thus apparent that over the range of pH common to each, the characteristics of typical pH/time curves are different. The rate of fall in horse longissimus dorsi, after a slow rate initially, is relatively fast; this slow phase is much less marked in ox longissimus dorsi and is virtually absent in the pig.

Fig. 2



Comparative rates of pH fall during post mortem glycolysis in typical l. dorsi muscles of horse (●), ox (▲) and pig (O) at 37°C under moist nitrogen. Mean of 20 observations. (Zero time = 1 hr. post mortem).

The data from which the mean curve was prepared illustrated another aspect of variability in pig longissimus dorsi muscle. Even under identical experimental conditions, glycolysis times between pH 6·7 and pH 5·5 ranged from 180 to 460 min. Further reference to this phenomenon will be made below.

Some biochemical criteria of glycolysis in longissimus dorsi, diaphragm and psoas muscles of the pig are compared in Table 1, from which it is clear that struggling at death lowers the initial pH of the latter two muscles to a greater extent than that of longissimus dorsi. Their relatively high ultimate pH suggests they have a lower glycogen store immediately ante-mortem and, by calculation from the initial pH, the initial glycogen level and the buffering power, this view is confirmed. The data are expressed in Table 1 as "glycogen at pH 7·2," on the assumption that this pH is near to the value in vivo. It is interesting to note that in the horse also the ultimate pH of psoas and diaphragm is significantly higher than that of the longissimus dorsi; in this case, however, a considerable quantity of glycogen remains unattacked by the glycolytic enzyme system at the ultimate pH (Lawrie, 1955). The significance of such phenomena in relation to the physiological inhomogeneity of glycogen has been considered recently (Lawrie, Manners and Wright, 1959). The actual in vivo glycogen concentrations will be somewhat higher than those given in Table 1 since, in pig muscle generally, glycogen is converted into glucose at a not inappreciable rate by amylolytic activity, operating from the moment of death (Sharp, 1958). This process will deplete glycogen and help to account for its absence at the ultimate pH.

TABLE 1

CHARACTERISTICS OF GLYCOLYSIS IN TYPICAL PIG MUSCLES

Mean of 6

Characteristic	*Muscle*		
	Diaphragm	*L. dorsi*	*Psoas*
Initial* pH..	6·36 ± ·12	6·71 ± ·04	6·20 ± ·15
Ultimate pH	5·99 ± ·08	5·45 ± ·02	5·75 ± ·18
Initial* Glycogen .. .. (mg/100 gm)	175 ± 50	628 ± 50	340 ± 117
Residual Glycogen .. .. .. (mg/100 gm)	20 ± 20	Nil	Nil
Buffering Power .. .. (10^{-5} equivs/pH/gm)	4·16 ± ·13	5·91 ± ·05	5·00 ± ·05
Glycogen at pH 7·2 .. .. .. (mg/100 gm) (Calculated)	455	973	745

* = at 1 hour post mortem.

The in vivo levels of glycogen in pig longissimus dorsi, psoas and diaphragm probably reflect the relative capacity of these muscles to gain energy by anaerobic or oxidative processes, lower levels being associated with a relatively high oxidative capacity and vice versa (Lawrie, 1953b, 1955). It is interesting to observe that the total acid soluble phosphorus contents of longissimus dorsi, psoas, diaphragm and heart in the pig also appear to bear an inverse proportionality to their presumed capacity for oxidative energy production (Table 2). The data for horse, ox and sheep follow the same sequence (Table 2). It may be pointed out that cytochrome oxidase activity in horse muscles does, in fact, increase in the order longissimus dorsi, psoas, diaphragm and heart (Lawrie, 1953c). In respect of the broad relative functions of these four muscles, at least as judged by the criterion of phosphorus content, there thus seems to be no major difference between pig muscles and those of the other three domestic species.

TABLE 2

TOTAL SOLUBLE PHOSPHORUS IN CERTAIN MUSCLES OF MEAT ANIMALS

mg./g

Species	*Muscle*			
	l. dorsi	*psoas*	*diaphragm*	*heart*
ox*	1·77 ± ·06	1·65 ± ·03	1·46 ± ·09	1·35 ± ·08
Horse**	1·91 ± ·06	1·43 ± ·02	1·41 ± ·02	1·27 ± ·02
Pigs**	2·04 ± ·04	1·93 ± ·05	1·47 ± ·04	1·11 ± ·03
Sheep**	1·75 ± ·04	1·69 ± ·07	1·33 ± ·03	1·03 ± ·02

* = mean of 20 samples. ** = mean of 24 samples.

Onset of Rigor Mortis

As pointed out above, the pH/time curves of pig and ox longissimus dorsi do not exhibit a marked initial slow phase like that of the horse. Since the rate of pH fall is controlled by the rate of ATP breakdown (Needham, 1942), it could be postulated that the latter was being offset by a mechanism replenishing ATP which operated more effectively in the horse than in the other two species. This view is at least superficially supported by the data in Table 3, which records that the initial level of CP/P, a means of reforming ATP from ADP, is approximately 19 per cent, 13 per cent and 7 per cent of the total soluble phosphorus in longissimus dorsi muscles of horse, ox and pig respectively. Since the onset of the fast phase of rigor mortis is associated with a rapid fall in the level of ATP/P (Bate-Smith and Bendall, 1947, 1949), it might be anticipated that the characteristic times to onset in the longissimus dorsi of these three species would be proportional to the initial store of CP/P. This is certainly the impression from Table 3, the times being 238, 163 and 50 min. in horse, ox and pig respectively. When, however, initial CP/P is plotted against time to onset of rigor mortis for a number of mammalian muscles (Fig. 3) it is evident that, in pig longissimus dorsi the pre-rigor delay is about half what would be expected from the general relationship between these two variables. Fig. 3. is compiled from data of Lawrie, (1953a), Howard and Lawrie (1957) and present work. Horse diaphragm is aberrant from the relationship in the opposite sense, for rigor mortis is delayed for much longer than would be anticipated from the initial CP/P level, but the discrepancy can be explained in this muscle by its glycolysis characteristics (Lawrie, 1953a). That the onset of rigor mortis in pig

TABLE 3

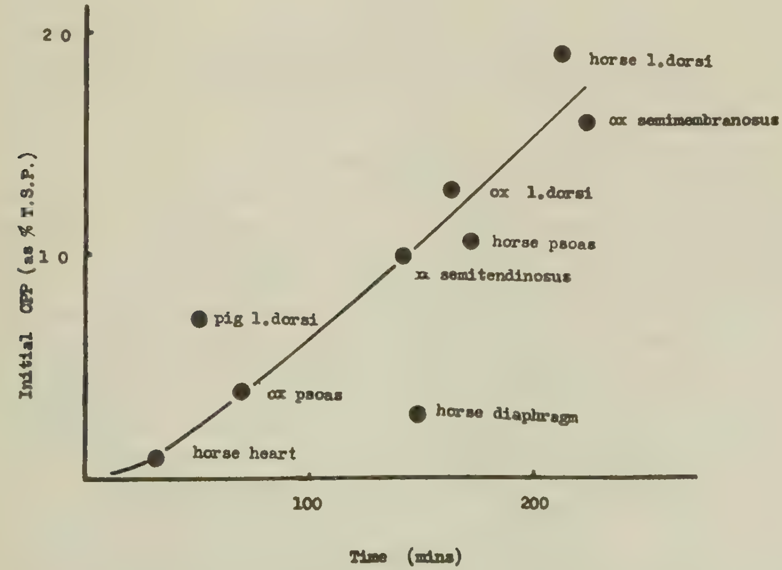
COMPARATIVE DATA ON POST MORTEM GLYCOLYSIS IN TYPICAL L. DORSI MUSCLES OF DIFFERENT SPECIES

Species	*Time to onset of fast phase of rigor mortis* ($min/37°/N_2$)	*pH*			*ATP/P (as % TSP) At onset*	*CP/P (as % TSP)*	
		*Initial**	*At onset*	*Ultimate*		*Initial**	*At onset*
Horse (6)**	238 ± 21	6·95 ± ·03	5·97 ± ·05	5·51 ± ·03	8·3 ± 0·5	18·9 ± 2·1	1·0 ± 1·0
Ox (13)***	163 ± 16	6·74 ± ·05	6·07 ± ·05	5·50 ± ·04	13·2 ± 0·6	13·2 ± 1·8	2·5 ± 1·0
Pig (6)	50 ± 12	6·74 ± ·01	6·51 ± ·08	5·57 ± ·04	21·0 ± 1·3	7·2 ± 1·4	2·8 ± 1·0

* i.e. at 1 hour post mortem. ** Calculated from Lawrie (1953) and unpublished data. *** Calculated from Howard and Lawrie (1956).

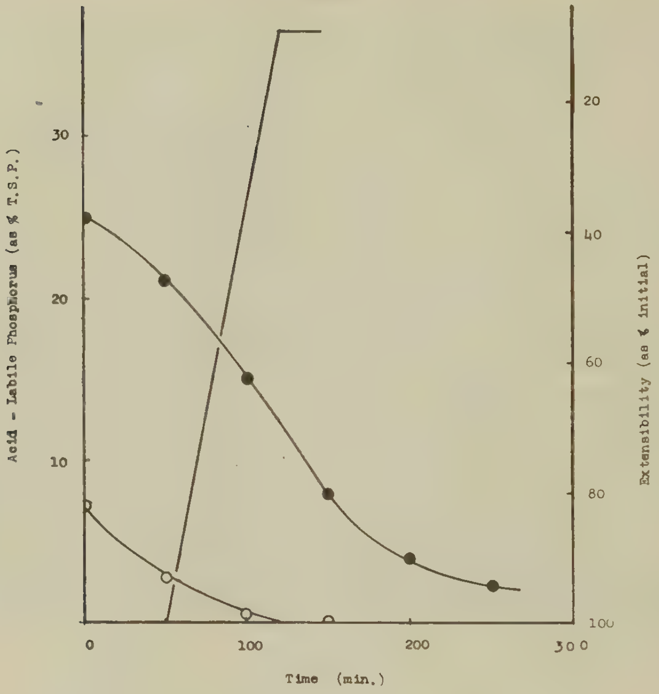
longissimus dorsi has further unusual features is also indicated by the fact that at onset both the level of ATP/P and the pH are considerably higher than in the longissimus dorsi of ox or horse (Table 3), although the level of CP/P is similar in all three species. The level of ATP at onset in pig longissimus dorsi is 87 per cent of its initial value. In the longissimus dorsi of the horse (Lawrie, 1953a) and ox (Howard and Lawrie, 1956) corresponding values are 30 per cent and 60 per cent respectively; and in the psoas of the rabbit, 60 to 65 per cent (Bendall, 1951). The general relationship between changes in extensibility and those in ATP/P and CP/P during the onset of rigor mortis in pig longissimus dorsi is shown in Fig. 4. It is clear that the level of ATP/P has fallen little when a swift loss of extensibility begins. Since, by the method used in the present work, a proportion of the acid labile phosphorus of hexose diphosphate and triosephosphates would be liberated during 10 min. hydrolysis in N HCl at 100°, such esters, if present in any quantity, would give erroneously high values for ATP/P, and perhaps account for the atypically high levels of the latter at onset. Nevertheless, when corrections were applied for the presence of interfering phosphate esters by the procedure of Bailey and Marsh (1952), the ATP/P values at the onset of the fast phase of rigor mortis, as thus amended, were still much higher than in the other species. This merits further investigation.

Fig. 3



The relationship for various mammalian muscles between initial level of creatine phosphate phosphorus (CPP) expressed as a percentage of total soluble phosphorus (TSP), and time (mins. at 37°C under moist nitrogen) to onset of the fast phase of rigor mortis.

Fig. 4

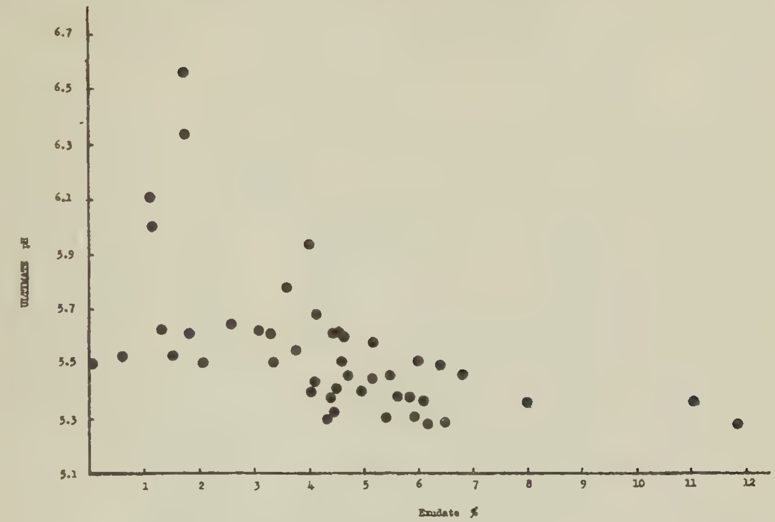


The onset of rigor mortis in l. dorsi muscle of the pig: changes in ATP/P, CP/P and extensibility as a function of time under moist nitrogen at 37°. zero time= 1 hr. post-mortem. Curves represent mean observations from six animals. ATP/P and CP/P expressed as % T.S.P. ●—●, ATP/P; O—O, CP/P; ——, extensibility.

Exudative Phenomena in Normal Longissimus Dorsi Muscles

Mammalian skeletal muscles will tend to exude more fluid at an ultimate pH of 5·3 to 5·5 than at in vivo values, since this range is near the isoelectric point of the principal constituent proteins, the actomyosin group. For a series of pig longissimus dorsi muscles the relationship between ultimate pH and the degree of exudation occurring during post mortem glycolysis under nitrogen at 37° is shown in Fig. 5. The data appear to conform reasonably well with the above view. It will be observed, however, that the relationship becomes asymptotic at pH 5·3 to 5·4; in this respect the relationship between ultimate pH and the degree of drip arising on thawing frozen muscle is similar (Bouton, Howard and Lawrie, 1957). At least one factor additional to ultimate pH is involved, however, in fluid retention. The work of several investigators has implied that there is a connection between the capacity of muscle proteins to hold fluid and the rate of post-mortem glycolysis, which, in turn, is determined by the rate of breakdown of ATP (Needham, 1942). Thus, Bendall and Marsh (1951) showed that the marked exudation

Fig. 5



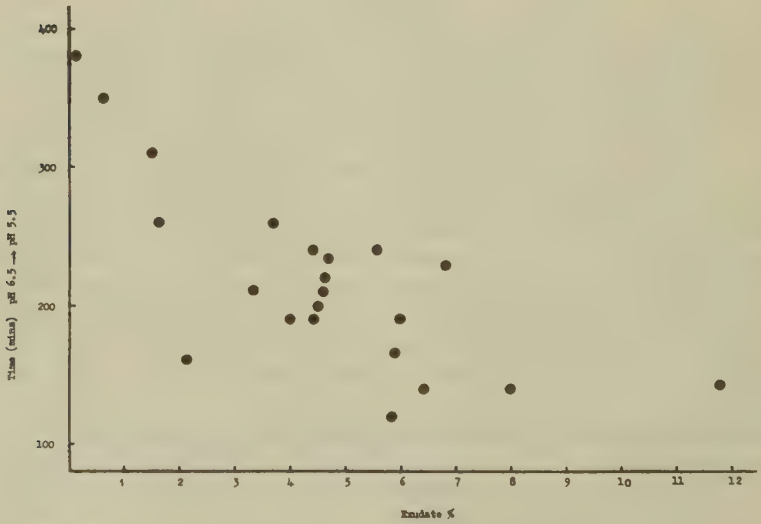
The relationship between ultimate pH and degree of exudation (as % initial wt.) during post mortem glycolysis (at 37° under N_2) in pig l. dorsi muscle.

occurring in muscles frozen before rigor mortis and then thawed (thaw rigor) is caused by a very high rate of breakdown of ATP. Marsh (1952) demonstrated that the degree of synaeresis of muscle breis was a direct function of ATP breakdown rate, the effect being independent of pH level, and Sharp and Marsh (1953) recorded that when large blocks of whalemeat go into rigor mortis at 37°, at which temperature the rate of glycolysis is high, as opposed to the lower temperatures at which this process usually takes place, they may submerge in their own exudate. Post-mortem glycolysis between the same fixed pH points has been shown above to proceed at markedly different rates, even under identical conditions (37° moist N_2), in the longissimus dorsi muscle of the pig. The degree of exudation arising in these circumstances was thus plotted against the time taken for the pH to fall from 6·5 to 5·5. The relationship is represented in Fig. 6, from which it is evident that while a slow rate of pH fall is associated with greater fluid retention, a fast rate of glycolysis tends to be associated with a greater degree of exudation. This finding accords with the implications of the experimental data of other investigators. Since the ultimate pH reaction is the same, pH 5·5, for all rates of pH fall, the effect of this factor on fluid retention can here be disregarded.

Biochemical Characteristics of White Muscle Disease

It would seem that even normal pig longissimus dorsi muscle has a variable tendency to exude fluid. Nevertheless the extent of exudation is much less than that observed in pigs exhibiting the white muscle condition. In accounting for the abnormally wet, pale musculature characteristic of this syndrome, it is necessary to implicate both the level of ultimate pH and the rate of pH fall. Ludvigsen (1954) deduced that the latter must be exceedingly fast, since at 30 min. post mortem a pH of 5·4 was found, and this has been confirmed by Wismer-Pedersen (1959). It appears that the low initial pH is not determined by an abnormally low value immediately prior to death. From the relationship noted in the present study, a large degree of exudation would be expected in these circumstances. Owing to the relatively low incidence of severely exudative muscle in the United Kingdom and the consequent difficulty, under commercial circumstances, of selecting affected carcases for detailed examination—with attendant depreciation—immediately after slaughter, it has been feasible so far to study rates of pH and of ATP fall in only a few psoas muscles. These muscles are easily accessible, being exposed on removal of the abdominal contents. In white exudative psoas muscle it was found that the ATP/P level 40 min. post-mortem was 10 per cent, of the TSP: the corresponding value in this muscle, when normal, was over 20 per cent. The psoas, however, although it is more susceptible to the stress of enforced exercise (Callow, 1936), seems relatively insusceptible to the condition. Longissimus dorsi and semimembranosus muscles have been found to show the most frequent and striking effects.

Fig. 6



The relationship between time taken to fall from pH 6·5 to pH 5·5 and degree of exudation (as % of initial wt.) during post mortem glycolysis (at 37° under N_2) in pig l. dorsi muscle.

Lack of pigmentation in exudative longissimus dorsi muscle is associated with diminished buffering power, viz. $5{\cdot}66 \times 10^{-5}$ equivs./

pH/gram compared with about $5{\cdot}9 \times 10^{-5}$ normally (Table 1), and a lower content of total acid soluble phosphorus of 1·4 to 1·8 mg/g. in comparison with about 2·0 mg/g. normally (Table 2). The low phosphorus content of exudative muscle was also observed by Henry *et al.* (1955).

In the present work, the white muscle condition has generally not been detected until 18 hours post-mortem, when more extensive exposure of the musculature is commercially possible. The implication of the ultimate pH level as a factor contributing to exudation is clear from Table 4, which records data on appearance and ultimate pH in rectus femoris, semimembranosus and longissimus dorsi muscles from two groups of Landrace pigs. Although the categories "M.D." and "normal" have been used, the latter merely serves to distinguish musculature which was superficially pink and non-exudative from that which was obviously affected. The term "normal" has here clearly a different significance from that used in relation to the Large White/Middle White breeds. For these, as has been shown, an ultimate pH of 5·5 in the longissimus dorsi is characteristic and a value of 5·35 would be unusually low (Table 4: Group A). As judged by the mean level of ultimate pH in M.D. pigs, and by the difference between it and that in corresponding normal animals, the semimembranosus and, even more, the longissimus dorsi muscles are mainly affected. This accords with the results of Ludvigsen (1954). The rectus femoris is relatively little involved. It is pertinent to note, however, that the rectus femoris in M.D. pigs, while not having the characteristic pale appearance, is nevertheless exudative.

The mean ultimate pH values of 5·11 and 5·14 respectively in the longissimus dorsi and semimembranosus muscles of affected pigs in Group A are remarkably low, (Table 4) and individual values of 4·94 and 4·78 have been found for these two muscles. That lactic acid production appears to account for such low values is indicated in Fig. 7 which shows that the relationship between this substance and ultimate pH is continuous from pH 6·5 to 4·9. The lowest ultimate pH value recorded in Danish Landrace pigs affected by "muskeldegeneration" is 5·1 in the longissimus dorsi muscle, the mean being 5·3 (Ludvigsen, 1957a). The French workers report a mean ultimate pH value of 5·24 in exudative muscles of the hind limb (Henry *et al,* 1955). Since the muscles they regard as normal have a mean pH of 5·95, the exudation observed could, however, be explained in terms of ultimate pH level alone without invoking any abnormality of glycolysis rate. In this context, unlike Ludvigsen (1954) and Wismer-Pedersen (1959), they find no difference in the pH soon after death between exudative and non-exudative muscles. Differences in ultimate pH of this order could be found in normal pigs in which muscle glycogen had been more or less depleted by fatigue pre-slaughter.

Although, in a relative sense, the musculature of the pigs in

TABLE 4

CHARACTERISTICS OF CERTAIN MUSCLES FROM "NORMAL" AND "M.D." PIGS

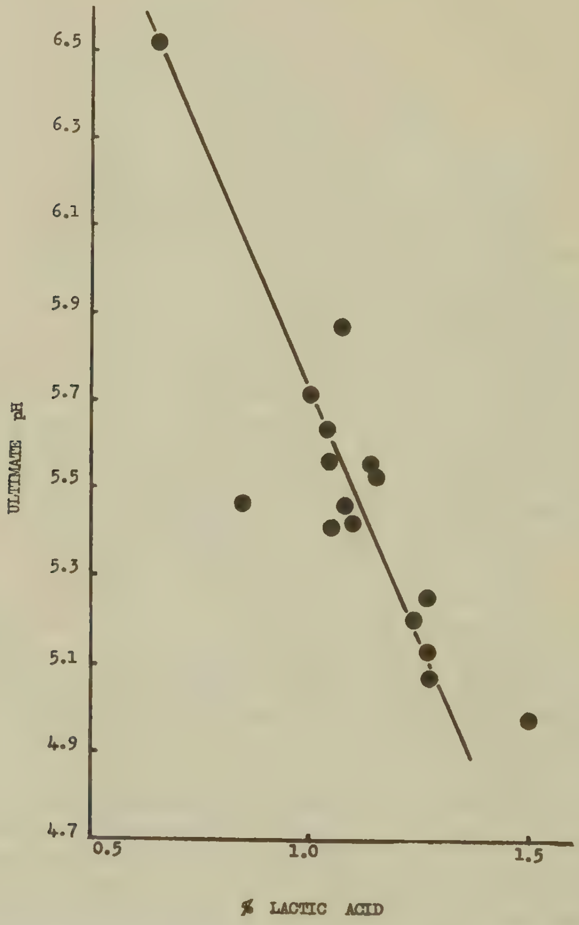
Characteristic	*Muscle*					
	Rectus femoris		*Semimembranosus*		*Longissimus dorsi*	
	Group A					
	"*Normal*"	"*M.D.*"	"*Normal*"	"*M.D.*"	"*Normal*"	"*M.D.*"
Ultimate pH	5·56 ± ·04 (10)	5·45 ± ·03 (10)	5·44 ± ·02 (10)	5·14 ± ·05 (10)	5·35 ± ·03 (10)	5·11 ± ·05 (10)
Colour	Pink	Pink	Pink	White	Pink	White
Condition	Dry	Exudative	Dry	Exudative	Dry	Exudative
	Group B					
	"*Normal*"	"*M.D.*"	"*Normal*"	"*M.D.*"	"*Normal*"	"*M.D.*"
Ultimate pH	6·13 ± ·21 (4)	5·62 ± ·13 (4)	5·84 ± ·24 (4)	5·40 ± ·11 (4)	5·80 ± ·27 (4)	5·30 ± ·04 (4)
Colour	Pink	White	Pink	White	Pink	White
Condition	Dry	Exudative	Dry	Exudative	Dry	Exudative

Group B could also be classified as "M.D." or "normal", the ultimate pH levels for all muscles in both categories were markedly higher than those in Group A (Table 4). In the first place this indicates a relative deficiency of muscle glycogen in the former; but, what is perhaps more significant, it substantiates the view that the level of ultimate pH is not the sole factor determining exudation. This was marked at a mean ultimate pH of 5·62 in the rectus femoris of "M.D." carcases in Group B, whereas in the "normal" rectus femoris in Group A no exudation was noticed at a mean ultimate pH of 5·56. Exudation was considerable in one rectus femoris even at pH 5·92. The phenomenon is reflected at lower pH levels in semimembranosus and longissimus dorsi muscles (Table 4). Further support for the importance of rate of pH fall, in determining fluid holding capacity, is found in the data of Howard and Lawrie (1957): the semimembranosus muscle of an insulin-tetanized steer exuded considerable fluid despite an ultimate pH of 6·18, but its rate of post-mortem glycolysis was unusually fast.

The distribution of ultimate pH values in an exudative longissimus dorsi from a Landrace pig is shown in Table 5 together with the mean data from five normal longissimus dorsi muscles of Large White x Middle White pigs. It will be seen that the ultimate pH in the lumbar region of the exudative longissimus dorsi is very much lower than that in the thoracic region, although values in the latter are nevertheless lower than at corresponding levels in normal longissimus dorsi. Exudation in the lumbar region of the affected longissimus dorsi was severe; in the thoracic region it was slight. It will also be seen from Table 5 that there is a gradation of pH in the affected longissimus dorsi, values becoming lower in proceeding caudally. In the normal longissimus dorsi muscles, however, no such gradation is manifest. This is unexpected since it is found in the longissimus dorsi muscles of normal steers, mean values being 5·53 ± ·01 (45) in the region of the 8th, 9th and 10th rib, for example, and 5·46 ± ·01 (45) in the region of the 4th, 5th and 6th lumbar vertebrae (Howard and Lawrie, 1956, 1957). No immediate reason for the greater involvement of the lumbar region of the longissimus dorsi in M.D. pigs can be suggested. It may not be without significance, however, that the former is the later developing part during growth (Hammond, 1932); and, in this same connection, the semimembranosus, which is apparently more susceptible to the condition than the rectus femoris, is a later developing muscle than the latter, (Hammond, 1932).

Of the muscles of the hind limb the semimembranosus is the most susceptible to depigmentation and exudation, but it may not be affected uniformly. The surfaces of semimembranosus and semitendinosus muscles, exposed by a planar cut normal to the limb, revealed two zones. The musculature in the more central of these had a normal pink colour and an ultimate pH of 5·56: only 1 cm. distant, in the peripheral zone, the musculature was white, exudative and had a pH of 4·94. The delineation between these zones was abrupt; yet it appeared to correspond to no anatomical division of the muscles.

Fig. 7



The relationship between ultimate pH and percentage of lactic acid in pig l. dorsi muscle.

Non-uniformity in exudative pork muscle has been referred to recently in U.S.A. (Briskey, Bray, Hoekstra, Phillips and Grummer, 1959); and it is of interest that non-uniformly pale and exudative musculature should be predominantly found in Landrace pigs (Judge, Cahill, Kunkle and Bruner, 1959). A less striking degree of inhomogeniety in non-exudative muscle, and in the pH range normal for Large White/Middle White pigs, has been studied by Scaife (1955) and Hornsey (1959). Low pH values were associated with relative absence of pigmentation, the latter being confirmed chemically. Likewise in the present study, determinations of pigment have indicated its virtual absence from pale muscles. Wismer-Pedersen, (1959), on the other hand, found no systematic difference in pigment content between light and dark zones of exudative muscle.

TABLE 5

ULTIMATE pH OF NORMAL AND EXUDATIVE L. DORSI MUSCLE FROM THE PIG

Location (vertebra)	*Normal Mean (5 sides) S.E.*	*Exudative*
3rd Thoracic	— —	5·52
4th ,,	— —	5·43
5th ,,	5·52 ± ·03	5·34
6th ,,	5·51 ± ·04	5·34
7th ,,	5·50 ± ·03	5·28
8th ,,	5·48 ± ·04	5·31
9th ,,	5·49 ± ·04	5·30
10th ,,	5·51 ± ·04	5·28
11th ,,	5·50 ± ·03	5·32
12th ,,	5·51 ± ·03	5·40
13th ,,	5·52 ± ·03	5·31
14th ,,	5·52 ± ·02	5·28
1st Lumbar	5·54 ± ·02	5·18
2nd ,,	5·49 ± ·03	5·25
3rd ,,	5·49 ± ·03	5·16
4th ,,	5·49 ± ·03	5·09
5th ,,	5·49 ± ·03	5·13
6th ,,	5·51 ± ·03	5·13

Histological Aspects of Exudative Pig Muscle

The unusual superficial characteristics of white, exudative muscle are reflected at the histological level. The degree of abnormality appears to be related to the ultimate pH. For example, in the case of longissimus dorsi muscles at pH 5·1, although the fibres were still parallel and showed cross striations, the interior of the majority was considerably disrupted (Fig. 8a): the effect may be compared with normal fibres of pig longissimus dorsi muscle at pH 5·5 to 5·6 (Fig. 8b).

At pH 4·9 all fibres had an abnormal appearance (Fig. 9). Some were straight and showed clear longitudinal markings; others were both twisted and finely corrugated and there were frequent breaks and spaces between fibres. It should be pointed out that these samples went into rigor mortis whilst attached to the skeleton and that any shortening observed in the histological sections does not arise merely because the muscle was not under tension. Corrugation has been described by Ludvigsen (1954) and Henry *et al.* (1955), although, in the photomicrographs published by the latter workers, the general aspect resembles that of normal muscle fixed during rigor mortis. In such the amplitude of corrugation is of the same order as fibre width, whereas it is clear from Fig. 9 that the corrugations have a much smaller relative amplitude. Although similar corrugations can be obtained in sections of normal muscles under certain conditions of fixing and staining, all fibres would be corrugated in such circumstances. On the other hand, as Fig. 9 indicates, corrugated fibres are here interspersed with straight ones.

Both Danish and French workers commented on the absence of cross striations from exudative muscle, and in a preliminary account of the present findings, their absence in straight fibres at pH 4·9, was reported (Lawrie, Gatherum and Hale, 1958). Closer examination under higher magnification has shown that straight fibres do have cross striations (Fig. 10), but these are closer and finer than in corrugated fibres and are so obscured by the general opacity of the straight fibres as to be normally undetectable. The former have, in a given distance, about twice as many cross striations. It thus appears that the corrugated fibres have not shortened but have been passively twisted by the shortening of adjacent straight fibres. The general appearance resembles that found in normal muscle when rigor mortis has been precipitate (Bendall, J. R.: personal communication). In the process, both isotropic and anisotropic bands have diminished to approximately the same extent. A cross section of exudative semimembranosus muscle indicated that the intrafibrillar contents had shrunk away from the sarcolemmae: this feature again accords with a precipitate onset of rigor mortis.

DISCUSSION

The pale, exudative musculature found in certain pigs of the Landrace breed in the United Kingdom is apparently similar to that described by Danish investigators (Ludvigsen, 1954, 1955; Wismer-Pedersen, 1959). Although the incidence of the condition has so far been low in this country, the ultimate pH values which have been found suggest that there can be a greater severity of attack. It is of particular interest that exudative muscle should have been found at a relatively high ultimate pH. This clearly emphasizes the involvement of factors other than pH level in controlling the degree of exudation. That the rate of pH fall during post mortem glycolysis is an important additional factor is further indicated from the data on the normal longissimus dorsi muscle of the pig—defined as having an initial pH of 6·7 and an ultimate pH of 5·5. Even under identical conditions of temperature and anaerobiosis, the times taken by such muscles to pass between the same fixed points of pH can vary nearly threefold, and the degree of exudation bears an inverse relation to these times. It could thus be inferred that the rate of pH fall is particularly fast in these muscles which show the white exudative condition: the nature of the present histological data accords with this view, and it aligns with the work of Ludvigsen and Wismer-Pedersen on "muskeldegeneration". On the other hand certain features of the phenomena described by the French workers could be explained on the basis of ultimate pH differences alone.

The origin of the pigs' susceptibility to the white muscle condition invites speculation. A large number of diverse manifestations of muscular dystrophy are known, many of these being associated with depigmentation and exudation (Adams, Denny-Brown and Pearson, 1953). Considerable attention has been given by Blaxter, Watts and Wood (1952) to a muscular dystrophy found in calves which is superficially characterised by lack of pigmentation and is attributable to vitamin E deficiency. Photomicrographs of muscle from vitamin E deficient calves show hyaline nodes and proliferation of the sarcolemma (McDonald, Blaxter, Watts and Wood, 1952): these histological features distinguish the condition from white muscle disease in pigs and indeed from another superficially similar dystrophy which also occurs in calves (Billon and Henry, 1958). Histologically, the latter more closely resembles the porcine condition. On the other hand, twisting and corrugation similar to that observed in pig musculature (Fig. 9 and 10) are regarded by Bicknell and Prescott (1942) as characteristic of that form of muscular dystrophy in man which arises from vitamin E deficiency.

Common to the composition of white exudative muscle in calves and pigs are the lower potassium and higher sodium concentrations (Blaxter and Wood, 1952; Henry *et al.*, 1958; Billon and Henry, 1958): there is a correspondingly low level of sodium and a high level of potassium in the blood (Ludvigsen, 1954). Concomitantly, as the marked exudation indicates, extracellular fluid increases and intracellular fluid diminishes. Another common feature is, of course, the absence of myoglobin from the muscle. Nevertheless, only in the white muscle condition studied in calves by Blaxter and Wood (1952) is myoglobinuria mentioned. The latter is suggestive of a fairly acute onset, as in paroxysmal paralytic myoglobinuria in horses, which is also associated with vitamin E deficiency (Biörck, 1949). That myoglobinuria was not a noteworthy feature of the other white muscle conditions indicates that these are of a more chronic nature and that the abnormally low myoglobin content of these muscles probably originates long before the immediate antemortem period. It is conceivable that this circumstance could signify reversion of the muscle structure to a more anaerobic type, such being characterised by the relative absence of myoglobin and by a greater capacity for gaining energy from lactic acid production (Lawrie, 1953b). This would, in turn, make more difficult the provision of energy for the intracellular retention of potassium and water. The initiation of this reversion could be a diminished general stimulus for oxidative metabolism, arising from vitamin E deficiency as in the calf (Blaxter and Wood, 1952) or low thyroid activity as postulated in the pig (Ludvigsen, 1955). Ludvigsen (1955, 1957b), however, has associated the loss of potassium from the intracellular environment with the white muscle syndrome in a more positive sense. He has suggested that by breeding Danish Landrace pigs for a higher rate of weight increment and for increased body length, animals have been selected in which the pituitary gland has an enhanced content of growth stimulating hormone, and a reciprocal deficiency of ACTH and thyroid stimulating hormone. His views are supported by the prophylactic effects achieved by administering iodocasein 10 days before slaughter, and by cortisone prior to stress producing circumstances. In pigs of this kind the hyperkalaemia arising during the alarm phase of the reaction to stress would thus not be easily counteracted (Selye, 1947, 1950), and an uncompensated lowering of potassium concentration in the musculature would lead to loss of fluid containing myoglobin. The work of Henry *et al.* (1958) supports the hypothesis that the white muscle condition is a typical "disease of adaptation" (Selye, 1944). It has been shown that when susceptible pigs are exposed to even such minor stress as confinement in cages for a week there is a marked negative potassium balance, which may be prevented by ATCH administration.

As the present data indicate, the muscles of the normal pig tend to have a relatively small store of glycogen. Even very moderate activity in the immediate preslaughter period could deplete it sufficiently to give a high ultimate pH (Callow, 1936, 1937). It would have been supposed that in pigs which were especially susceptible to stress, the ultimate pH would frequently have been very high. Yet animals affected by the white muscle condition have a distinctly sub-normal ultimate pH in their musculature. This indicates that glycogen reserves have not been appreciably depleted before death. Although pigs tend to have a low initial pH through struggling at slaughter, the lactic acid produced would remain within the muscles and contribute to the ultimate pH, blood circulation having ceased. The primary deficiency in the affected pigs is thus not of 17-hydroxycorticoids for the restitution of carbohydrate reserves: the hyperkalaemia in white muscle disease, to which several investigators have referred, suggests rather a deficiency of 11-deoxycorticoids. The unusual ionic relationships resulting within the muscle cells, thereby, could conceivably affect the mechanism which normally prevents glycolysis below pH 5·5 and, by permitting further glycogen breakdown to lactic acid, account for the low ultimate pH values attained by affected muscles. Alternatively, the latter may arise through the breakdown of a structually abnormal glycogen elaborated in affected muscles. It is known that the structure of glycogen may change because of definite physiological factors (Cori, 1953) even within a given muscle (Lawrie *et al.*, 1959).

The high degree of variability in the pig, as evidenced by the scatter of initial pH values, the differing glycolysis times in normal longissimus dorsi muscles and the short range heterogeneity of pigmentation and ultimate pH in both normal and dystrophic musculature, distinguishes this animal from domestic species such as ox and horse. Even in the absence of a selection policy as intensive as that applied to the Danish Landrace, which Ludvigsen has cited in accounting for susceptibility of the latter to the white muscle condition, it is conceivable that in other breeds, such as Large or Middle White, the effects of domestication have been more marked than with oxen and horses since the period between generations in the pig is relatively much shorter. This circumstance may well have predisposed pig musculature to exhibit unusual physiological features which, while less spectacular than massive exudation, are nevertheless also attributable to directed development over many generations. On the other hand the relatively high level of ATP/P at which the fast phase of onset of rigor mortis occurs in the longissimus dorsi of the pig is a feature more fundamental to muscle structure and may reflect some aspect of the energy production mechanism in this species. It will be obvious that there are numerous problems in comparative physiology and biochemistry awaiting investigation.

CONCLUSIONS

In the pig, normal longissimus dorsi muscles have been defined as those having a pH of about 6·7 at 1 hour post mortem and an ultimate pH of about 5·5: typical pig muscles have considerably lower glycogen stores than those in horse and ox. The onset of the fast phase of rigor mortis in pig longissimus dorsi muscle occurs after 50 min. at 37° under moist N_2, when zero time is one hour post mortem. The initial CP/P is considerably lower and the level of ATP/P at onset considerably higher than in the longissimus dorsi of species studied hitherto. Nevertheless, as judged by total soluble phosphorus content, the relative functions of heart, diaphragm, psoas and longissimus dorsi muscles in the pig are similar to those in horse, ox and sheep.

At 37°C and under moist N_2 the times taken for normal pig longissimus dorsi muscles to pass from the same initial pH to the same ultimate pH vary threefold. There is an apparently inverse relationship between these times and the degree of exudation of fluid by the muscles: this is independent of the ultimate pH level. The massive degree of exudation associated with the white muscle syndrome thus indicates a very high rate of post-mortem glycolysis. This is supported by the low ATP/P levels in affected psoas muscles immediately after death and by photomicrographs of affected longissimus dorsi and semimembranosus muscles. The ultimate pH of affected musculature was very low, mean values being $5{\cdot}11 \pm{\cdot}05$ and $5{\cdot}14 \pm{\cdot}05$ respectively in longissimus dorsi and semimembranosus, individual values being as low as 4·78. Marked exudation at an ultimate pH of $5{\cdot}62 \pm{\cdot}13$ in affected rectus femoris muscles emphasizes the importance of rate of pH fall as a factor distinct from ultimate pH in determining water-binding capacity in muscles.

Lumbar regions of longissimus dorsi and peripheral regions of semimembranosus are especially susceptible to exudation and depigmentation.

The data have been compared with those described by French and Danish workers and the aetiology of the white muscle condition has been discussed.

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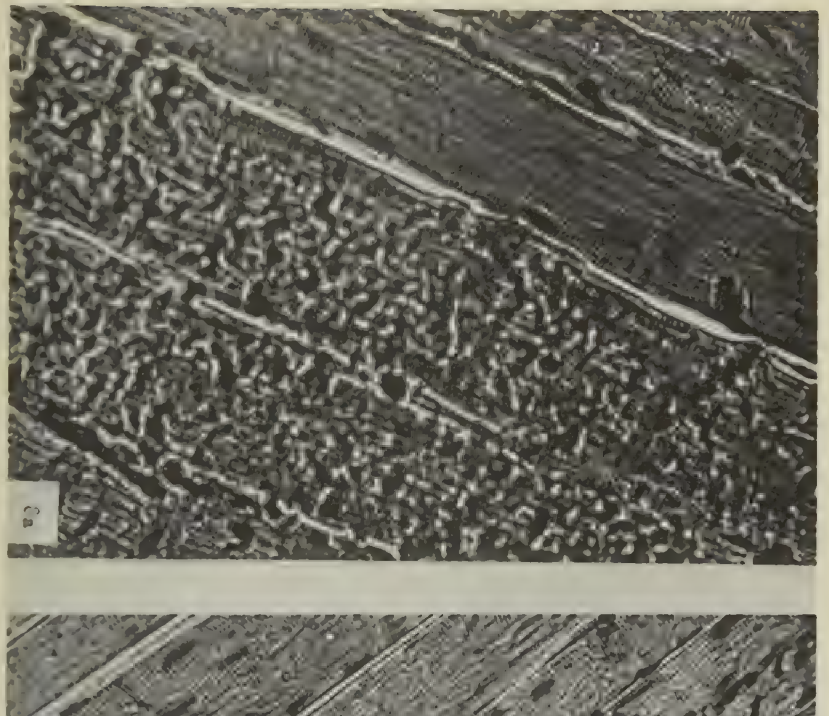
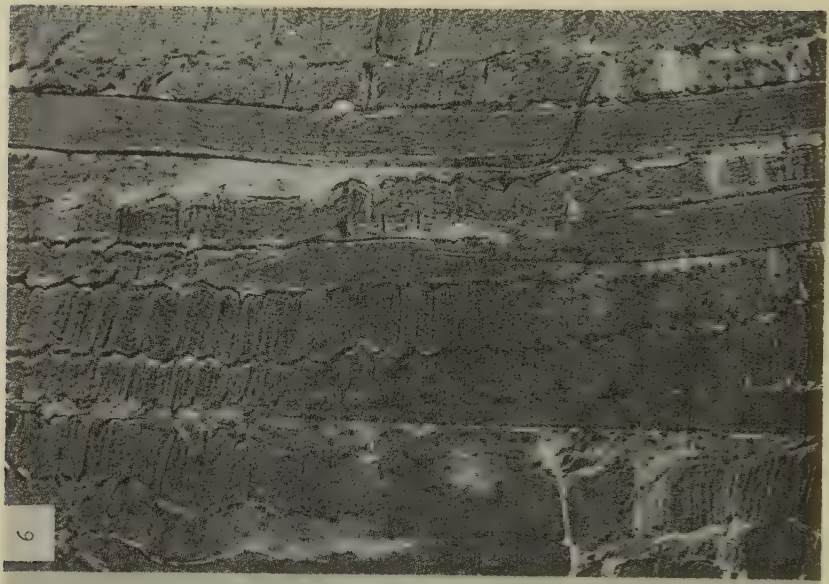


Fig. 8. (a) Longitudinal section of exudative l. dorsi muscle from pig, pH 5·13.
(b) Longitudinal section of normal l. dorsi muscle from pig, pH 5·5 to 5·6.
Fixed in formalin: celloidin embedded.
Stained: haematoxylin, Van Gieson. (× 180).



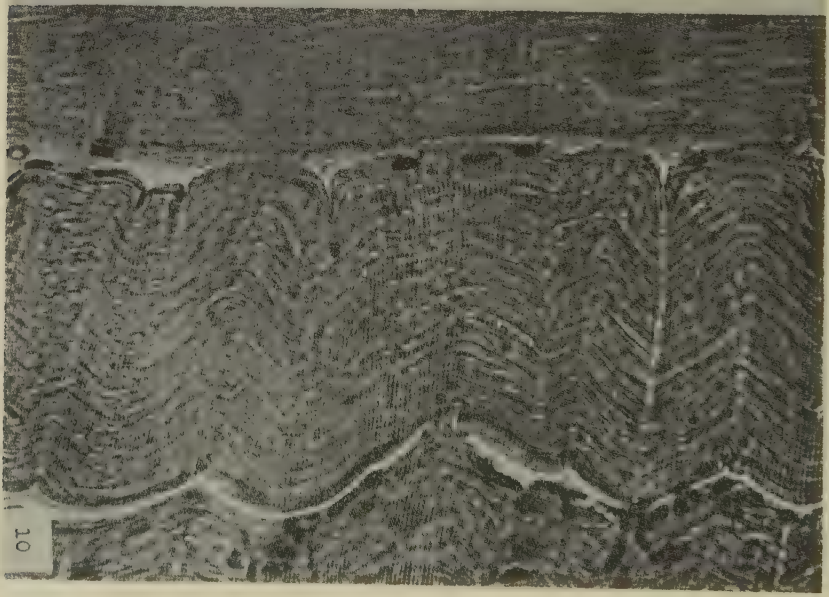


Fig. 9. Longitudinal section of exudative semimembranosus muscle of pig, pH 4·94. Note presence of both straight and corrugated fibres, and general abnormal disorientation of section.
Fixed in formalin: celloidin embedded.
Stained: haematoxylin, Van Gieson. (×43).

Fig. 10. As in Fig. 9 at higher magnification. Note apparent absence of cross striations in left hand fibre and twisting and corrugation of the other two.
Fixed in formalin: celloidin embedded.
Stained: haematoxylin, Van Gieson. (×180).

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MUSCULAR DYSTROPHY IN SHEEP AND NEONATAL MORTALITY

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INTRODUCTION

Muscular dystrophy and myopathy have frequently been described in lambs and young sheep.

Until recently reports of such diseases indicated that the majority of outbreaks were due to a true or conditioned vitamin E deficiency since vitamin E was effective both as a curative and prophylactic (see review by Blaxter and McGill, 1955). Recently, however, it has been suggested that myopathy can be caused by factors other than vitamin E deficiency. Bosanquet, Daniel and Parry (1956) have described a myopathy affecting male and female sheep, usually in middle age (2½ to 3½ years old), with an annual attack rate of about 10 per cent in the breeding females. They relate this myopathy to scrapie disease of sheep and suggest that it is probably an inherited disease. A genetical origin for a few isolated occurrences of muscular dystrophy has been previously postulated by Roberts (1929), Morley (1954) and Hartley and Dodd (1957) but new-born lambs alone were affected. In some areas of the U.S.A. white muscle disease has for many years occurred in lambs born of ewes fed on green crop feeds, especially legumes grown in certain areas. Since the characterising lesions simulated those of naturally occurring and experimentally induced avitaminosis E it was concluded that the disease was due to vitamin E deficiency. It is now found however, that sodium selenite when fed to supply 0·1 p.p.m. of selenium in the ration prevents the disease occurring, whereas vitamin E has no protective effect (Muth, Oldfield, Schubert and Remmert, 1959).

In the spring of 1958 lesions of muscular dystrophy were found on post-mortem examination in a number of the sheep at this Institute and since several features are of interest it is briefly reported here.

RESULTS

Sheep

The sheep were in several different groups: (a) purebred Suffolk ewes of all ages used for an investigation into a possible genetic factor in scrapie; (b) cast South Country Cheviot ewes, bought in and mated to Cheviot rams and the offspring used as experimental animals; (c) North Country Cheviot ewes mated to a Cheviot ram and maintained at the Institute for several years on a copper-deficient diet; (d) cast South Country Cheviot ewes mated with a Cheviot ram and used for magnesium metabolism work; (e) Blackface ewes of varying age mated to a Cheviot ram and fed on hay obtained from a "swayback" farm.

Diet

The dietary regimen varied a great deal from group to group. Most of the Suffolks (group a) were pastured all the year round on permanent grass fields away from the environment of the Institute, but a few were kept at Moredun. From the end of September until lambing time they were fed hay ad lib, the hay being obtained from a number of farms in the south-east of Scotland. Starting two weeks prior to tupping in October the sheep received a daily supplement of ½ lb. of "ewe nuts" which was increased gradually to 1½ lb. two weeks prior to expected lambing. Turnips were also fed towards the end of pregnancy but in no appreciable quantity. The cast Cheviot ewes in use for the production of experimental lambs (group b) were fed similarly and were confined to Institute ground; they had very little grass and were not fed as heavily with ewe nuts as the Suffolks. The North Country Cheviot ewes in the copper-deficiency experiment (group c) were housed and fed on a diet consisting of hay and oats (copper content 2 to 3 p.p.m. D.W.) obtained from an Aberdeenshire farm. Each ewe received 1½ lb. hay and 1 lb. oats each day until the end of pregnancy. Twelve of these sheep had been on this diet since October, 1955, when they were bought in as gimmers. Six of the twelve received 100 mg. copper each week in addition to the hay and oats. Since there had been a high incidence of muscular dystrophy (predominantly myocardial) in the 1956 lamb crop (Nisbet, Butler and Macintyre,

TABLE 1

MYOPATHY IN THE VARIOUS GROUPS OF SHEEP

	No. of ewes examined	*No. with dystrophy*	*No. of lambs examined*	*No. with dystrophy*
(Group a) Purebred Suffolks (Scrapie +)	3	1		
Purebred Suffolks (Scrapie —)	9	9	20	9
(Group b) Cast Cheviot ewes (Scrapie —)	10	4	Nil	Nil
(Group c) Cheviot ewes on copper-deficient diet (Scrapie —)	4	4	8	1
(Group d) Cheviot ewes on magnesium metabolism work (Scrapie —)	5	0	3	2
Blackface ewes on "sway-back hay" experiment	Nil	Nil	7	Nil

1959) a vitamin E supplement* was added to the above diet from December, 1956, onwards. This supplied about 70 mg. α-tocopheryl acetate per day for the first month but thereafter was increased to 120 mg./day. The lambs born in 1957 and 1958 were also given 70 mg. α-tocopheryl acetate by mouth twice weekly from the age of three days. There was no muscular dystrophy in the 1957 lamb crop.

The cast Cheviot ewes on the hypomagnesemia experiment (group d) were also housed and fed hay ad lib and about ¼ lb. of ewe nuts per sheep per day during pregnancy and early lactation after which the diet was suddenly replaced by freshly cut grass from a heavily fertilised grass plot.

The Blackface ewes (group e) were left indoors and fed hay ad lib obtained from a farm where swayback constantly occurs; ewe nuts were also fed.

Clinical History

Suffolks (group a). The foundation stock of 110 weaned ewe hoggs was bought in 1955 from fourteen different flocks. This stock was divided into three flocks according to whether the hoggs were from scrapie-affected parents (Flock A) or from farms where scrapie was not known to be present (Flocks B and C). There was no direct contact between the flocks except for some interchange of rams at mating time between Flocks A and B. One shepherd was responsible for the three flocks. Apart from the lambs born in these flocks nineteen ewe hoggs were added to Flock A and ten adult ewes to Flock B during the years following 1955.

In the lambing seasons of 1956 and 1957 there was in this group a total loss of 24 lambs, most of which were caused by abortion of undetermined etiology. During the lambing of 1958 losses were very much higher. Prior to lambing, two ewes were lost, one with metritis resulting from a decomposing foetus in utero and the other due to eversion of the gravid uterus. During lambing time there were four cases of dystokia; in three the lambs had died and decomposed in utero and in one the lamb was normal but dead when born. A further 119 ewes and gimmers lambed at full-term, but of the 199 lambs born 67 did not survive, a mortality rate of 33 per cent. Of the 65 lambs which died within a week, 25 were born dead and 22 died within 24 hours of birth, many of the latter only living for a few minutes. The percentage losses in Flocks A, B and C were about equal. Of three sets of triplets involved, only one lamb survived; of 30 sets of twins involved both lambs died in 17 and one lamb in 13 cases; 12 single lambs died.

Since the majority of lost lambs were born dead or died shortly after birth, few or no symptoms were seen. The lambs born alive but dying immediately after birth were unable to stand, being prostrate and flabby although big, well-formed, full-term and with no apparent abnormality. There were no "rotten" or premature lambs. The ewes did not retain their foetal membranes, which macroscopically showed no abnormality such as is seen in most types of infectious abortion. Of the twenty lambs which survived for more than 24 hours and died later, all were weak and recumbent at birth although of good body size and of these twelve, although kept in the incubator, quickly died without showing any other symptoms. Of the rest, two had convulsions and died on the second and third days, one had inco-ordination of gait and frequently stood on its forelegs, dying on the third day, another refused to suck and was hyperaesthetic while several had flexed fetlocks at birth and/or were unable to raise their heads. In addition to the animals which died, a number of lambs which survived had varying degrees of flexion of the fetlocks and some were unable to lift their heads to the proper position. Such symptoms persisted for weeks or months.

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* "Rovimix E, 10%" with a silica base supplied by Roche Products Ltd.

The number of ewes involved in these losses was 45 representing 39 per cent of all lambing sheep. The ewes were in good bodily condition, not overfat, but many had a protracted parturition, making little or no effort to expel the lamb or lambs. A few had prolapsed vaginas before or after lambing. Nine of these ewes died or were killed in extremis during the lambing period. The remaining ewes showed no obvious ill-health.

Cast Cheviot ewes (*group b*). A hundred and twenty ewes had been purchased at tupping time and were used for the production of experimental lambs. They all lambed normally and apart from the fact that four ewes developed abdominal hernia both ewes and lambs remained healthy. Ten of these sheep were killed for various reasons during the lambing period and examined postmortem.

Ewes on copper deficiency experiment (*group c*). Three out of twelve Cheviot ewes in this experiment died from dystokia with metritis and prolapse during the spring of 1958 and another died from old-standing lesions of fascioliasis. The remainder showed no signs of ill-health during this period but three were barren. Three lambs born from two of the ewes with dystokia were born dead but a lamb from the third dystokia case survived as did a further eleven lambs. The surviving lambs were killed for experimental purposes when they were 6 to 8 weeks old.

Ewes on magnesium experiment (*group d*). Seven out of 30 cast Cheviot ewes died from various causes during May and June but none of the deaths was associated with difficulties at lambing time; some deaths were expected as a result of the experimental procedure. Three lambs were born dead and five died during April and May. There were 37 healthy lambs.

Ewes on "swayback hay" (*group e*). The ten Blackface ewes produced seven lambs, both ewes and lambs were healthy until the latter were killed at six to seven weeks of age.

As can be seen, the losses were greatest amongst the Suffolk sheep and if these had not occurred it is probable that little notice would have been taken of the minor troubles in the other experimental animals.

Post-mortem Examination

Suffolks (*group a*). Twelve Suffolk ewes were examined post-mortem, 8 of which were the mothers of dead or weak lambs. It is not proposed to describe in detail the post-mortem findings since all died from causes which were well established, or were killed; eight died from metritis following dystokia (one also had advanced scrapie), two died of scrapie and two were killed as healthy controls (one had lambed normally and the other had had a dead lamb). The most interesting feature of the post-mortem examinations, however, was the finding of lesions of muscular dystrophy which were readily recognised when the musculature was examined carefully. In all but one of the dystokia cases, dystrophy was present in the abdominal muscles, which in three cases had ruptured with the weight of the gravid uteri. With the exception of one of these cases, dystrophic lesions were also present in other skeletal muscles of the body, e.g. latissimus dorsi, semitendinosus, extensor carpi radialis, subscapularis, intercostal, gluteus, biceps brachii etc. Dystrophic lesions were not found in the two uncomplicated cases of scrapie, neither of which had borne a lamb during the year. Dystrophy was found however, in both healthy controls which were killed.

Of the 65 lambs which were born dead or died soon after birth, twenty were thoroughly examined post mortem, both macroscopically and histologically. In eleven of these no abnormality could be found but lesions of muscular dystrophy were present in the skeletal muscles in nine. Only one mother of these twenty lambs died. It was examined post mortem and both ewe and lamb showed lesions of dystrophy. No pathogenic organisms were isolated from these lambs.

Cast Cheviot ewes (*group b*). Of the ten ewes killed from this group, four had lesions of muscular dystrophy in various skeletal muscles; the lesions were not extensive. The internal abdominis obliquus and external gluteus profundus, rhomboideus, vastus lateralis and brachialis were some of the muscles involved.

Since the number of lambs lost in this group was negligible no lambs were examined post mortem.

Ewes on copper-deficient diet (*group c*). The four ewes in this experiment which died were examined post mortem and all four showed lesions of muscular dystrophy; the muscles of two were extensively involved, e.g., rhomboideus, latissimus dorsi, medial head of triceps, trapesius, semimembranosus, rectus femoris etc. Unfortunately it was not possible to examine the lambs which were born dead. One lamb, the offspring of a healthy ewe, died when five days old and showed extensive lesions of muscular dystrophy, both cardiac and skeletal muscles being affected. The remaining seven healthy lambs showed no dystrophic lesions when killed at six to eight weeks of age.

Ewes on magnesium experiment (*group d*). Four of the 30 ewes died during the lambing period for reasons unassociated with parturition. One ewe which had given birth to dead lambs was killed and examined. None of these ewes showed lesions of muscular dystrophy but muscle lesions were present in two of the three Cheviot lambs born dead. No bacterial pathogens could be isolated.

Ewes on "swayback hay". None of the seven lambs killed showed lesions of muscular dystrophy.

TABLE 2

MUSCULAR DYSTROPHY

Tocopherol Content of Livers

Ref. No.	*Dystrophy*	*Age*	*Breed*	*Group*	*Total tocopherol content mg./100 g. FW*	*Diet*
14/58	—	4 wks.	Cheviot	c	0·85	Hay and oats supplemented with vitamin E. Rovimix E supplying about 100 mg. α-tocopheryl acetate/day fed to ewes for the last 16 months. The lambs received this supplement (≡ ~70 mg. α-tocopheryl acetate) twice weekly from the age of 3 days.
17/58	—	6½ wks.	Cheviot		1·02	
18/58	—	7 wks.	Cheviot		0·65	
20/58	+	5 days	Cheviot		1·07	
1858	+	Ewe-4 yrs.	Cheviot		1·23	
1859	+	Ewe-4 yrs.	Cheviot		1·87	
826	+	6 wks.	Cheviot	d	0·49	Hay from various sources and sheep nuts. No vitamin E supplement.
H 122	+	1 wk.	Suffolk	a	0·72	
H 126	+	1 yr.	Cheviot	b	0·53	
5/58	—	6 wks.	Blackface x Cheviot	e	2·35	
6/58	—	6½ wks.	Blackface x Cheviot		1·45	
7/58	—	6 wks.	Blackface x Cheviot		1·23	
S 99 (H113)	+	Gimmer	Suffolk	a	1·07	
C 375	+	Ewe-5 yrs.	Cheviot	b	1·00	
H 109	+	Gimmer	Suffolk	a	0·35	
H 110	+	Gimmer	Suffolk	a	0·78	
H 111	+	Gimmer	Suffolk	a	0·61	
H 112	+	Ewe-3 yrs.	Suffolk	a	0·72	

Pathology

The lesions found in the muscles had no specific features, being mainly soft, pale, poorly defined foci, although on occasion the lesions were made more conspicuous by the presence of haemorrhages and/or chalky, white streaks. The lesions were generally relatively small and solitary but some were multiple in a muscle and a few had coalesced to form irregular, pale patches occupying up to two-thirds of a muscle.

Histological examination of all lesions showed that they were those of muscle degeneration similar to those previously described elsewhere (Hulland, 1959). They were indistinguishable from the lesions caused by a vitamin E deficiency.

Tocopherol Content of Liver Samples

The tocopherol content of the livers of a number of sheep examined post mortem is shown in Table 2. Some had very definite lesions of muscular dystrophy whilst others showed none.

DISCUSSION

Although the present investigation gives no definite evidence as to the aetiology of the neonatal mortality described it appears possible that it may be related in some way to the occurrence of muscular dystrophy. It is clear however that muscular dystrophy was not the direct cause of death or weakness in many of the lambs since it was not found in some of the lambs at post-mortem examination. It could be postulated that the finding of muscular dystrophy in so many of the ewes which were examined post mortem was an indication that the normal muscular processes of birth were interfered with and that this delayed birth sufficiently to cause death or anoxia of the lamb. The clinical history of protracted and "lazy" births would support such a hypothesis. On the other hand, the fact that a few lambs were born dead without either themselves or their mothers showing lesions of muscular dystrophy when examined post mortem makes such an interpretation difficult to accept unless on occasion muscles could be functionally weak without having recognisable histological lesions of dystrophy. Probably the most rational explanation is that the perinatal mortality and the muscular dystrophy are the result of some undetermined factor. A number of bacterial, viral and protozoan infections are known to cause neonatal and perinatal mortality but none of these was found in the present instance. It is possible that some form of malnutrition was the cause of both the deaths in lambs and the muscular dystrophy. The most likely deficiency would seem to be vitamin E but since α-tocopheryl acetate in adequate amount fed to one group of sheep did not prevent the muscular dystrophy in either ewes or lambs, and since the tocopherol content of the livers of a number of both affected and unaffected animals showed no correlation with the absence or occurrence of lesions, it seems unlikely that an absolute deficiency of vitamin E was the cause of the present trouble. That the diets of the groups varied considerably also makes vitamin E deficiency unlikely. It could of course be postulated that the "environmental factor" is a virus, but up to the present no evidence for this has been obtained.

A peculiar feature of the present findings is that many ewes dead from a number of conditions or killed specifically for post-mortem examination should have extensive lesions of muscular dystrophy and yet during life show no symptoms of any kind or none specifically related to the dystrophy. This is in contradistinction to the findings of Parry (1957) who described similar lesions of myopathy and related them to the prominent clinical signs of scrapie. Although muscular dystrophy was found in one sheep affected with scrapie, the remaining sheep with dystrophy were not scrapied and in fact many of them were in groups of sheep unlikely ever to be affected. Further, five ewes with advanced scrapie, although not part of the present investigation, were examined post mortem during the same period and had no lesions of muscular dystrophy, a finding similar to that reported by Hulland (1959). The incidence of muscular dystrophy in the sheep kept at Moredun Institute seems to vary considerably from year to year. In the spring of 1956 there was a high incidence of muscular dystrophy in the lamb crop (Nisbet, Butler and Macintyre, 1959) which did not recur in 1957. In the spring of 1957 detailed post-mortem examinations of the muscles of considerable numbers of ewes, many affected with scrapie, showed little or no evidence of dystrophy. As described above a similar investigation on the same or similar groups of sheep in the spring of 1958 frequently showed the lesions of muscular dystrophy in all classes of sheep, although again such lesions were not always present in scrapied sheep. Further, not only does the appearance of muscular dystrophy differ from year to year, but it was obvious from continuing examinations during 1958 that the lesions in ewes seen so frequently during the early part of 1958 were not seen in similar sheep later in the year; it therefore seems probable that such lesions resolved during the summer months. This evidence would suggest that the muscle lesions are "environmental" in origin rather than genetic.

CONCLUSIONS

A considerable incidence of muscular dystrophy found in both ewes and lambs is unconnected with scrapie or vitamin E deficiency and the etiological factor could not be determined.

The relationship between the lesions of muscular dystrophy, the perinatal mortality and the peculiar dysfunction of the legs and necks of a number of the lambs which survived could not be ascertained.

The fact that over the last few years a considerable number of outbreaks of serious perinatal mortality in sheep have been reported throughout Britain from which no pathogens could be isolated makes further investigation of such losses as those described above very necessary.

ACKNOWLEDGMENTS

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EPENDYMOMA IN A COW

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INTRODUCTION

Apart from reports on nerve sheath tumours, descriptions of primary neoplasms of the nervous system in cattle are rare.

Gliomas, of unknown type, were recorded by Hjärre (1934) and Joest (1921); the latter's case affected the spinal cord. Three astrocytomas in cattle were reported by Stünzi and Perlstein (1958). In a survey of 636 tumours from domestic animals, Plummer (1956) discovered one brain tumour, an astrocytoma, in a heifer. Fankhauser (1947) described 2 glioblastoma multiforme in cattle, one of which occurred concomitantly with multiple meningiomata. A spongioblastoma was found by Barboni (1940) and in 1956 Luginbühl described a cerebral glioma that had features of both an astrocytoma and an oligodendroglioma. Other recorded neoplasms affecting the central nervous system of cattle include a medulloblastoma (Cordy, 1953) and meningioma (Fankhauser, 1948; Monlux, Anderson and Davis, 1956).

The purpose of this paper is to describe an ependymoma that occurred in a cow.

METHODS

Brain material was fixed in 10 per cent formalin and embedded in paraffin and celloidin. Stains used were haematoxylin and eosin, thionin, periodic acid-Schiff, Mallory's phosphotungstic acid haematoxylin (P.T.A.H.), van Gieson and a silver reticulin stain.

RESULTS

Case History

The subject was a recently purchased five-year-old Friesian cow. A fortnight after calving, it suddenly developed an abnormal gait with restricted movements of both limbs of the right side; it showed a tendency to lean against objects on that side and circled in an anticlockwise direction. The appetite remained good and there was no depression of milk yield (6 gallons daily). The animal appeared alert and hearing was unaffected, but there was some impairment of vision, accompanied by a sluggish pupillo-constriction reflex of the right eye. No evidence of positional nystagmus was present. The rectal temperature was 103°F and pulse rate 72/min. Laboratory investigations revealed normal serum calcium and magnesium levels (9·2 mg. and 2·1 mg./100 ml. respectively), and a neutrophilia (6,420 neutrophils/c.mm.); blood cultures were negative.

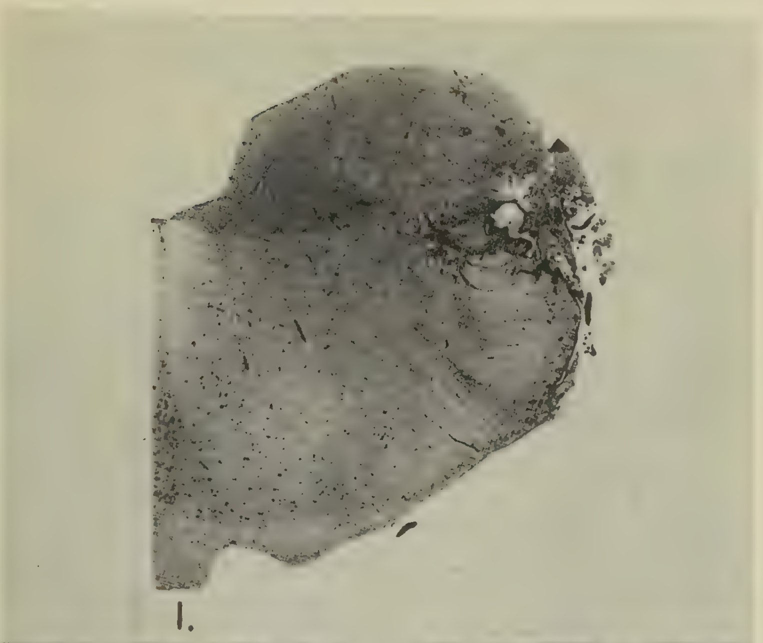
In view of the neutrophilia and slight fever the possibility was considered that these clinical signs could have been caused by an intracranial abscess and a course of penicillin therapy was carried out over a period of 4 days. There was no improvement. Over the next 6 months there was gradual deterioration; the animal eventually became recumbent on its right side. The cow was destroyed with intravenous chloral hydrate and an autopsy performed immediately. No abnormalities were found outside the central nervous system. A bright orange-yellow lesion occurred on the right side of the medulla occupying the region of the spinal vestibular nucleus. The area was triangular in shape and extended medially about 1 cm. from the lateral margin of the medulla, and was about 2 cm. in length.

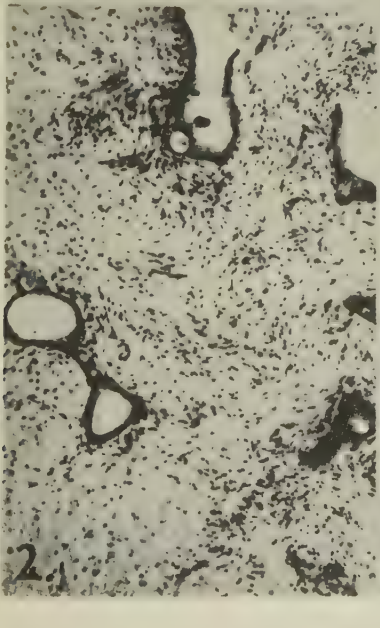
Histopathology

The tumour appeared to arise from the pia-arachnoid and invade the brain (Fig. 1) and its margins were ill-defined. It consisted of a system of tubules or canals between which were large numbers of proliferating astrocytes (Fig. 2). The canals were bounded with a cuboidal epithelium (Fig. 3) which, in sections stained with P.T.A.H., occasionally showed cilia and blepharoplasts. Nuclei were oval and vesicular, and mitoses were absent. In some places the continuity of the epithelium was broken and surrounding astrocytes appeared to spill into the tubules. The tubules also contained debris, lipid phagocytes and cholesterol clefts. Surrounding the canals were increased numbers of astrocytes and abundant glial fibres; some of the astrocytes were of the swollen gemistocytic type. In some regions the epithelium did not form tubules but appeared to extend in the form of sheets or papillae, the membrane forming a glove-like covering for a vascular tuft. Vessels throughout the neoplasm were large and thickened. This connective tissue also spread to contiguous parts of the tumour; elsewhere the stroma consisted of a glial fibre feltwork. Nervous tissue adjacent to the tumour showed some degenerative changes in the neurones, axons and myelin, but on the whole the normal tissue was well preserved despite the intimate presence of neoplastic tissue. In the region of the lateral vestibular nucleus, there was some thinning of the myelin and also evidence of a reactive gliosis. The latter also occurred caudal to the tumour in the region of the spinal vestibular nucleus.

DISCUSSION

There can be little doubt that this tumour is an ependymoma because of the ciliated epithelium and the presence of blepharoplasts: moreover, the formation of tubules or rosettes is fairly characteristic of these tumours in man (Russell and Rubinstein, 1959; Zülch, 1957). Curious features of the tumour in the cow are its invasiveness with sparing of much of the normal architecture, and the absence of a distinct boundary. Ependymomas in the cerebellopontine angle have been reported in man and it has been suggested that they arise from a projection of the ependyma through the lateral recess (Woltman, Kernohan and Adson, 1949).





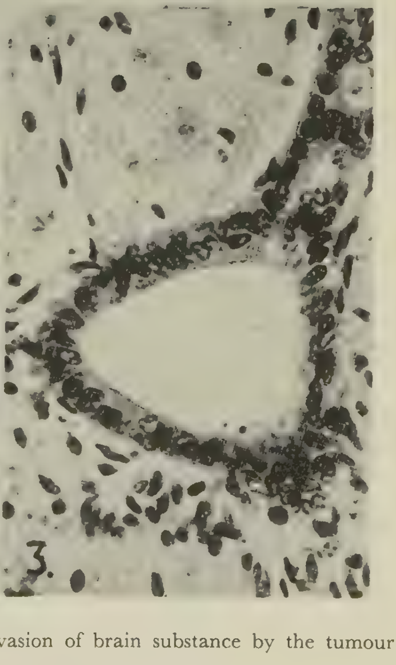


Fig. 1. Right side of medulla, showing invasion of brain substance by the tumour. Haematoxylin and eosin. × 4.

Fig. 2. Part of tumour showing the tubular formation, spilling of astrocytes into a tubule and gliosis. Haematoxylin and eosin. × 85.

Fig. 3. A tubule at higher magnification showing cilia. Haematoxylin and eosin. × 370.

CONCLUSIONS

An ependymoma is described in the hind-brain of a five-year-old cow.

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PATHOLOGICAL CHANGES IN THE BLOOD OF CHICKENS INFECTED WITH *SALMONELLA GALLINARUM*

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INTRODUCTION

It is generally believed that symptoms and death of individuals infected with *Salmonella* arise from the action of bacterial endotoxin on host tissues—the term endotoxin referring to the somatic antigen complex of these organisms (Boivin and Mesrobeanu, 1935). Latterly, attempts to define more precisely the mode of action in vivo of endotoxins have revealed a variety of physiological disturbances which may occur (Thomas, 1954). For example, the endotoxin of *Salm. typhi* acts on the autonomic system (Reilly, Compagnon, Tournier and du Buit, 1954) and that of *Salm. pullorum* causes marked alteration in the blood concentrations of glucose, urea and amino-acids (Dooley, Holtman and Jefferies, 1958). The pyrogenic effect of endotoxins has been investigated (Grant and Whalen, 1953; Atkins and Wood, 1955; King and Wood, 1958) and the clinical symptoms shown by rabbits subjected to continuous intravenous injection of endotoxin have included progressive leucopenia, hypothermia, cyanosis, respiratory distress and collapse (Landy, Skarnes, Rosen, Trapani and Shear, 1957).

Recent experimental data, emphasising as they do the importance and significance of polysaccharides as a cause of physiological dysfunction in the host, can be closely applied to the study of the pathogenesis of fowl typhoid. In this respect there are certain salient features of this disease which are of significance. Symptoms and death of birds are due to the ability of *Salm. gallinarum* to multiply rapidly in the host and it is only during the bacteraemic phase that the symptoms of acute infection can be observed. These may include greenish-yellow diarrhoea, loss of weight, inappetance and an increased thirst. Shortly before death, however, the symptoms are typical of a developing anoxia, as indicated by listlessness, weakness of the legs, cyanosis of the comb and wattles, an apparent state of coma with eyes closed, occasional respiratory gasping, sudden collapse and death. These latter symptoms may all occur within one to two hours before death. In many birds which become chronically ill the most noticeable symptoms are loss of weight and the development of a severe anaemia. A study of these observations in the light of recent research on the effect of high concentrations of polysaccharide in the body suggest that at a certain stage of infection, there is an interaction between the polysaccharide of *Salm. gallinarum* and circulating erythrocytes which may contribute to the cause of death of the bird as a result of anoxia.

It has been shown (Buxton, 1959) that under favourable conditions, circulating erythrocytes may become sensitised in vivo with polysaccharide derived from *Salm. gallinarum* and these sensitised erythrocytes are subsequently removed rapidly from the circulation. The following experiments were made to observe any significant changes in haemoglobin levels and fragility of erythrocytes consequent upon infection of chickens with *Salm. gallinarum*.

MATERIALS AND METHODS

The preparation of bacterial polysaccharide, sensitisation of erythrocytes and haemagglutination tests were carried out according to the techniques of Buxton (1959).

Haemoglobin estimations. Blood samples were obtained from the wing veins, using heparin as the anticoagulant. A volume of 0·2 ml. whole blood was added to 10 ml. distilled water, mixed, stood at room temperature for 30 minutes and then centrifuged for 20 minutes at 4000 r.p.m. Finally, 1·0 ml. supernatant was diluted with an equal volume of distilled water, giving a final dilution of 1/100. Readings were obtained on a "Unicam" spectrophotometer, using 10 mm. cells and a blue filter. After preliminary trials it was found that with blood samples derived from normal chickens of different ages the haemoglobin concentrations were so variable that final dilutions of blood from 1/50 to 1/100 were required in order to obtain satisfactory readings. These difficulties were overcome. For example, variations in the final concentrations of blood in water, which were used for spectrophotometric examinations, were allowed for in the final calculations for the values of "E" as recorded in the figures. The presence of lecitho-vitellin in blood from hens was frequently the cause of marked opacity, and before taking readings it was necessary to subject the sample to ether extraction.

Erythrocyte osmotic fragility tests. The technique employed was a slight modification of that described by Dacie (1956) which is based on the method used by Parpart, Lorenz, Parpart, Gregg and Chase (1947). A stock solution of buffered sodium chloride was obtained by dissolving NaCl (180 g.), Na_2HPO_4 (27·31 g.) and $NaH_2PO_42H_2O$ (4·86 g.) in 2000 ml. triple distilled water. A 1·0 per cent. solution in distilled water was prepared from this stock solution and the following dilutions were then made: 0·85, 0·75, 0·65, 0·60, 0·55, 0·50, 0·45, 0·40, 0·35, 0·30, 0·25, 0·20, 0·15, 0·10 and 0·05 per cent. solutions. Heparinised whole blood was added in volumes of 0·05 ml., by means of a capillary pipette, to tubes containing 5·0 ml. volumes of each dilution of salt solution and also to 5·0 ml. distilled water. The mixtures were shaken, stood at room temperature for 45 minutes and reshaken at intervals during this time. After centrifugation at 2000 r.p.m. for 10 minutes the supernatant from each sample was examined in an E.E.L. colorimeter. Recordings were taken using 10 mm. diameter tubes, a green filter (OGR1) and a blank obtained from the supernatant containing 0·85 per cent. NaCl.

RESULTS

Haemoglobin Estimations

Natural cases of fowl typhoid. Occasional mention has been made in the literature of clinical observations on the presence of anaemia in chickens severely infected with *Salm. gallinarum,* but no references have been found to precise measurements of haemoglobin levels in such birds. Figures recorded for normal haemoglobin levels in healthy chickens show considerable variation. For example, Olson (1937) quotes 11·76 g. per 100 ml. for cockerels and 9·11 g. per 100 ml. for hens. Moreover, he found that haemoglobin values in adult chickens vary with the seasons—the values being higher during winter months. Holmes, Pigott and Campbell (1933) reported that haemoglobin levels in chicks increased with age. During recent years improvements in methods of spectrophotometry have tended to lead to an increase in the accepted normal values for haemoglobin levels, and it has been stressed by Heilmeyer (1943) that the earlier accepted figure for man of 14 g. per 100 ml. should be amended to 16 g. per 100 ml.

A number of preliminary trials were made on blood samples from healthy chickens to obtain some data on normal haemoglobin levels as recorded by the technique outlined above. Two groups of adult chickens consisting of Rhode Island Red hens and Light Sussex cockerels were examined in this way and it was found that the haemoglobin levels for the hens varied from approximately 12 to 14 g. per 100 ml. and the figure for cockerels was in the range of 16 to 17 g. per 100 ml. Because of these variations, relatively small reductions in haemoglobin content resulting from bacterial infection would have little significance unless normal levels for the particular infected chickens were known.

In the first series of observations on infected birds, a number of hens were obtained from a natural outbreak of fowl typhoid. Three of these birds were suffering from diarrhoea and, although showing slight listlessness, blood samples were taken for haemoglobin estimations immediately on arrival and during the subsequent few days. One bird collapsed suddenly and died 23 hours after arrival. The second died 84 hours after arrival and the third survived. Haemoglobin estimations recorded from these three birds are reproduced in Figs. 1 to 3. Photometric readings were taken over the range 450 mμ to 650 mμ in order to observe changes in oxyhaemoglobin and methaemoglobin as well as in total haemoglobin.

Bird 1 was anaemic when first examined and possessed only 7·0 g. per 100 ml. haemoglobin. Twenty-three hours later, when in a state of collapse, the haemoglobin level had fallen to 3·38 g. per 100 ml. Bird 2 showed haemoglobin levels of 13·3 g. per 100 ml. on arrival and 12·6 g. 100 ml. 24 hours later. Eighty-four hours after arrival it had collapsed and died almost immediately after a blood sample had been obtained. This last sample showed a marked decrease in haemoglobin content (6·4 g. per 100 ml.) and in addition. the spectrophotometric readings showed evidence of methaemoglobin at 630 mμ (3·9 g. per 100 ml.). The figures obtained from bird 3, which recovered, are of particular interest in relation to the preceding results. On arrival this bird was extremely weak, diarrhoea was severe and both the comb and wattles were cyanotic. At this time the haemoglobin level was 4·8 g. per 100 ml. On the following day the bird showed distinct improvement—diarrhoea still persisted, but the comb and wattles no longer appeared cyanotic. At 36 hours after arrival the haemoglobin level had increased to 6·8 g. per 100 ml. and rose still further to 9·75 g. per 100 ml. after 96 hours, by which time clinical symptoms had practically disappeared.

Fig. 1

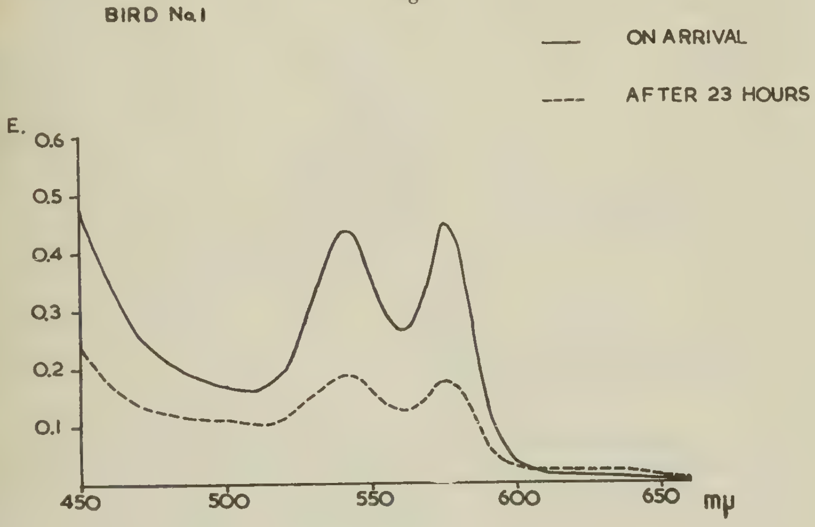


Fig. 2

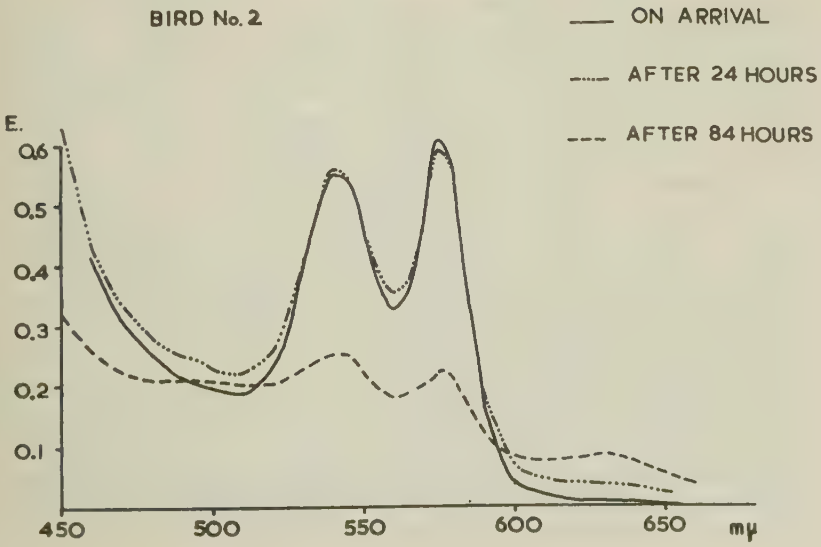
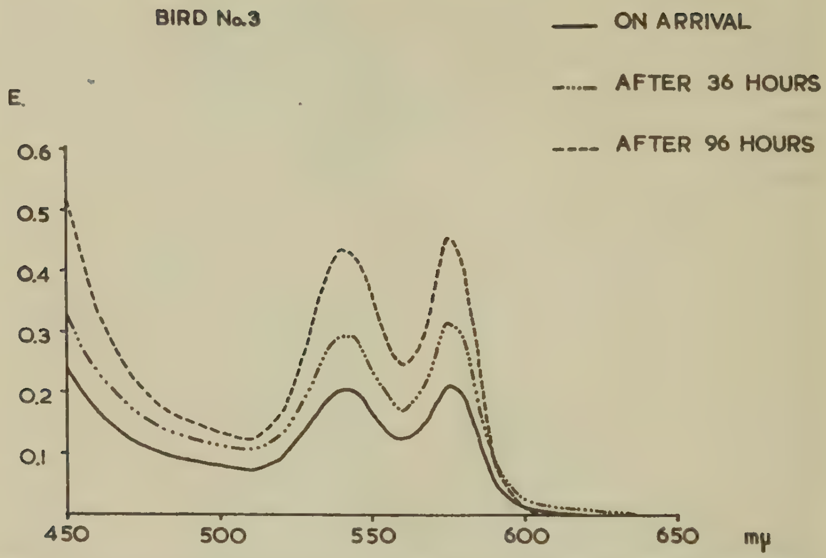


Fig. 3



Figs. 1–3 Showing fluctuations in spectrophotometric readings of haemoglobin from 3 naturally infected chickens.

The haemoglobin levels recorded from these birds are typical of results derived from similar observations made on other chickens which were naturally infected, and they emphasise several important features. The haemoglobin levels of infected chickens show little variation from normal until the bacteraemic phase has developed shortly before death in those birds which are fatally infected. Immediately before death, haemoglobin levels are extremely low and also at this time there may be traces of methaemoglobin. In those birds which recover from a severe and generalised infection haemoglobin levels will show a rapid rise, returning to approximately three-quarters of the normal level in about 4 to 5 days.

Experimental cases of fowl typhoid. The purpose of this experiment was to observe firstly, whether similar variations in haemoglobin levels to those recorded from naturally infected chickens could be reproduced under experimental conditions; and secondly, to identify more precisely the time after oral infection which elapsed before a marked reduction in haemoglobin levels occurred.

Four adult chickens, including two hens and two cockerels, were infected by mouth with an avirulent culture of *Salm. gallinarum.* From previous experience it was known that this strain produced a relatively mild generalised infection resulting in slight diarrhoea and loss of appetite for a period of about 48 hours. Thereafter, infected birds became clinically normal although they remained carriers for several months afterwards. On the 1st, 3rd, 6th, 8th, 10th and 14th days after infection blood samples were withdrawn from these chickens and examined. Only very slight changes were observed during the first 8 days after infection and the results obtained on the 1st, 8th, 10th and 14th days are recorded in Figs. 4 to 7. Fig. 8 shows the average fluctuations in haemoglobin levels for all 4 birds.

Fig. 4

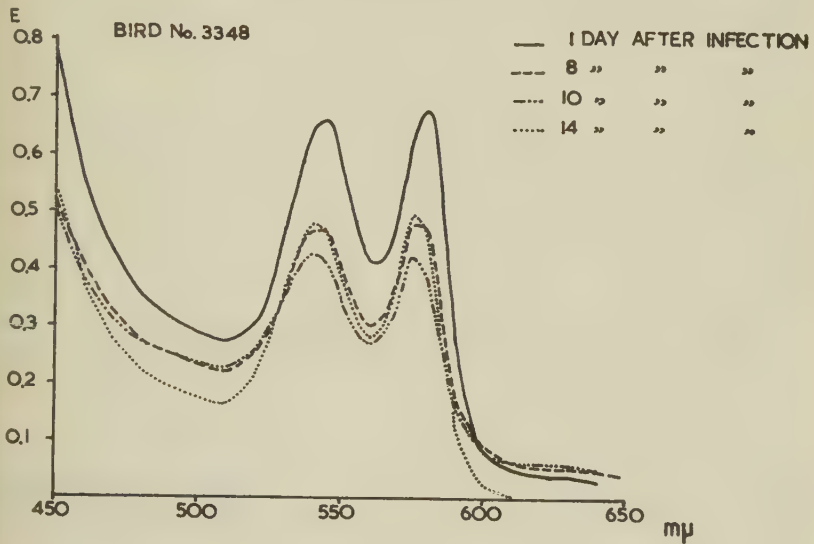


Fig. 5

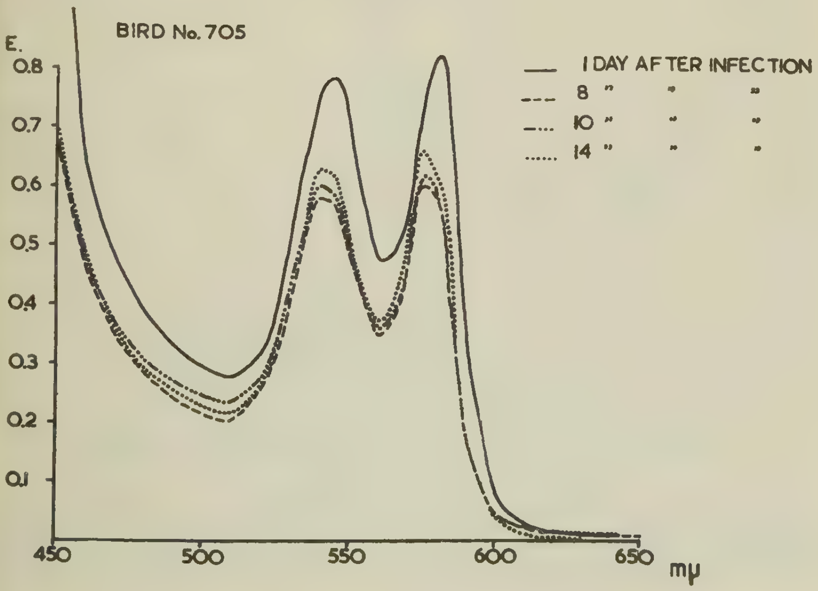


Fig. 6

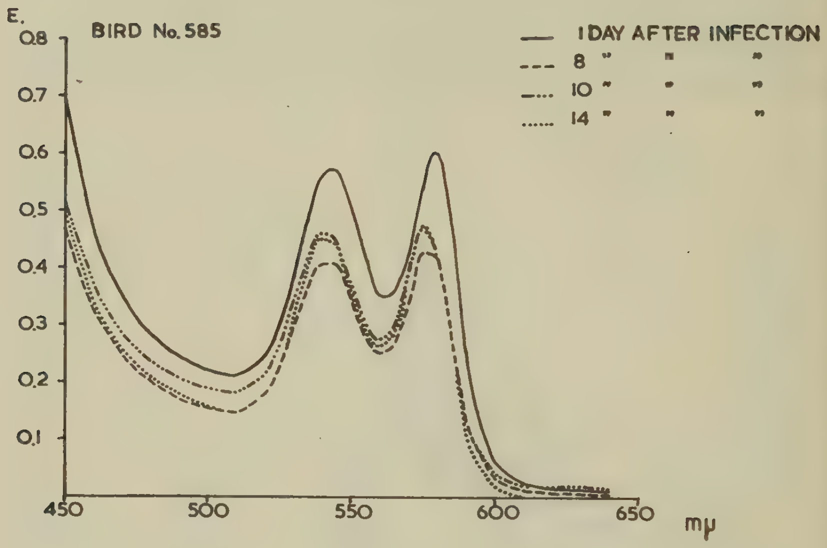
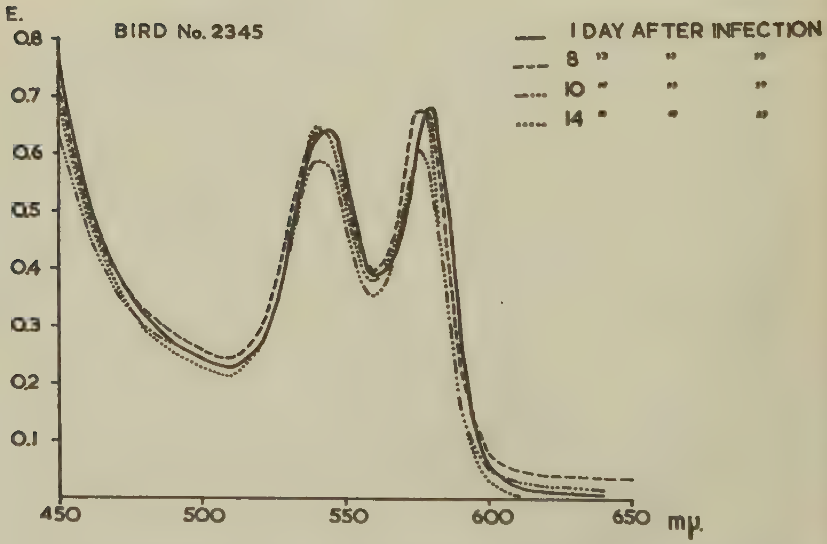


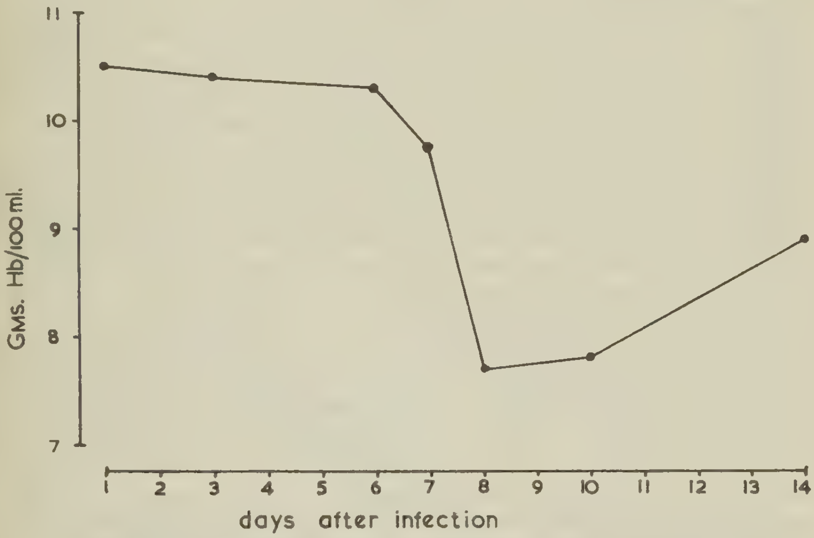
Fig. 7



Figs. 4–7. Showing fluctuations in spectrophotometric readings of haemoglobin from 4 chickens (Nos. 3348, 705, 585, and 2345) which survived oral infection.

It will be noticed that in 3 of these birds (3348, 705 and 585) on the 8th day after infection there was a marked drop in haemoglobin levels which began to rise again on the 10th and 14th days. In the case of bird 2345 the decrease was less marked and did not occur until the 10th day after infection. By the 14th day the level had returned to approximately the original concentration. Moreover, this bird showed no obvious clinical symptoms during the experiment, whereas the other three chickens suffered from some diarrhoea and loss of appetite during the 8th to 9th days of the experiment.

Fig. 8



Showing the average fluctuation of haemoglobin (g. Hb/100 ml.) from the recordings shown in Figs. 4–7.

The results described above are typical of those derived from other experiments. For example, infection of 12 adult hens with a strain of avirulent *Salm. gallinarum*, which produced no obvious clinical symptoms, did not give rise to any significant fall in haemoglobin levels. By contrast, haemoglobin levels in birds which survived severe infection but died a few weeks later were at a constant low level of 6 to 8 g. per 100 ml. during the chronic stage of infection. These results confirm the clinical signs of anaemia which have been occasionally described as a feature of the disease; and, moreover, they show that this anaemia is sudden in onset and occurs at about 8 days after oral infection, at a time when the infection becomes generalised and maximum invasion of the tissues is occurring.

Osmotic Fragility of Erythrocytes

In view of the coincidence of the stages of infection at which both in vivo adsorption of bacterial polysaccharide on to erythrocytes (Buxton, 1959) and the development of an haemolytic anaemia occur, it was considered that erythocyte fragility tests performed at this time might show significant variations from the normal which could be related to the pathogenesis of infection.

There is little information available on osmotic fragility tests performed on normal chicken erythrocytes. Many preliminary experiments have, therefore, been made on blood from normal chickens and the results obtained showed that the fragility of normal nucleated avian erythrocytes is similar to that of non-nucleate mammalian cells. By plotting percentage concentrations of NaCl against percentage haemolysis these results fell within the normal range for human erythrocytes as recorded by Dacie and Vaughan (1938) and show a slight decrease in fragility compared with the results recorded later by Dacie (1956). In a study of erythrocyte fragility in relation to haemolysis, Suess, Limentani, Darmeshek and Dolloff (1948), and Bolton (1949) have advocated recording results by plotting percentage concentrations of NaCl against percentage incremental haemolysis in each successive salt concentration. By this latter method it has been found that maximum haemolysis with avian erythrocytes occurs at salt concentrations of 0.35 to 0·4 per cent. In the majority of normal chickens the range of salt concentrations within which any degree of haemolysis occurs is 0·3 to 0·5 per cent., and occasionally "tails" to these single curves may occur at either of these limits of salt concentrations. By using this method of recording results it has been shown that in cases of haemolytic anaemia a characteristic binodal curve is obtained with mammals' blood (Suess *et al.*, 1948; Bolton, 1949). It was considered that similar results might be obtained from chickens infected with fowl typhoid in which anaemia is a clinical feature, and that the characteristics of these curves would give further information on the pathogenesis of infection.

After oral infection with Salm. gallinarum. A group of 6 adult hens was infected orally with a culture of *Salm. gallinarum* of reduced virulence. Subsequently, three of these birds showed no clinical symptoms of infection and erythrocyte fragility tests gave normal results during the ensuing four weeks. However, the remaining 3 birds showed clinical symptoms of infection during the 8 to 10 day period following oral dosage with *Salm. gallinarum*. Thereafter, they developed a reduced appetite, loss of weight and became clinically anaemic. The erythrocytes from these birds were tested for their fragility at intervals and the results are recorded in Figs. 9 and 10. Bird 148 died on the 14th day after infection, Bird 132 on the 20th day and Bird 655 survived. It will be noticed that in all three birds the normal curve for erythrocyte fragility became replaced by binodal curves which are characteristic of haemorrhage or haemolysis in mammals. The earliest appearance of these curves was on the 12th day after infection (Bird 148) and approximately 48 hours before death. Similarly, the binodal curve recorded from the blood of Bird 132 occurred on the 19th day after infection and about 24 hours before death. Bird 655 which survived infection showed a similar abnormal erythrocyte fragility on the 19th day and three days later this had returned almost to normal with a single peak at 0·4 per cent. concentration of salt.

Fig. 9

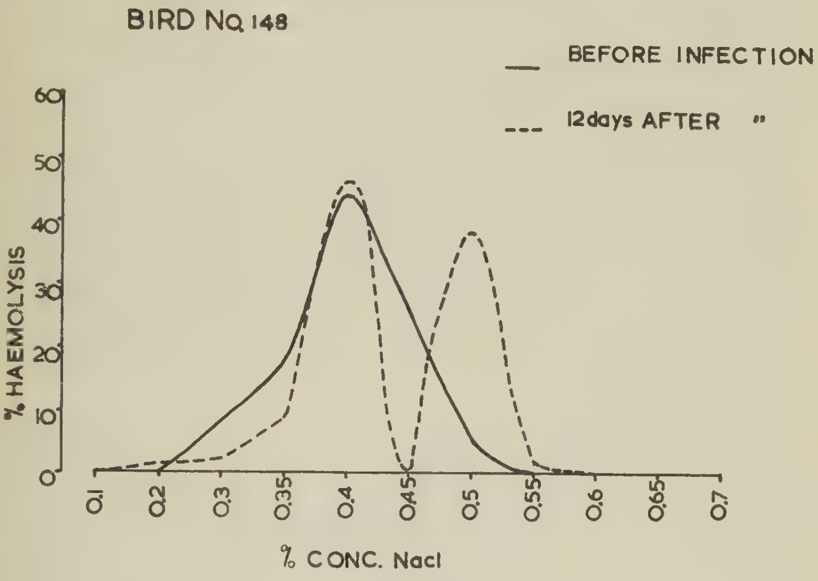
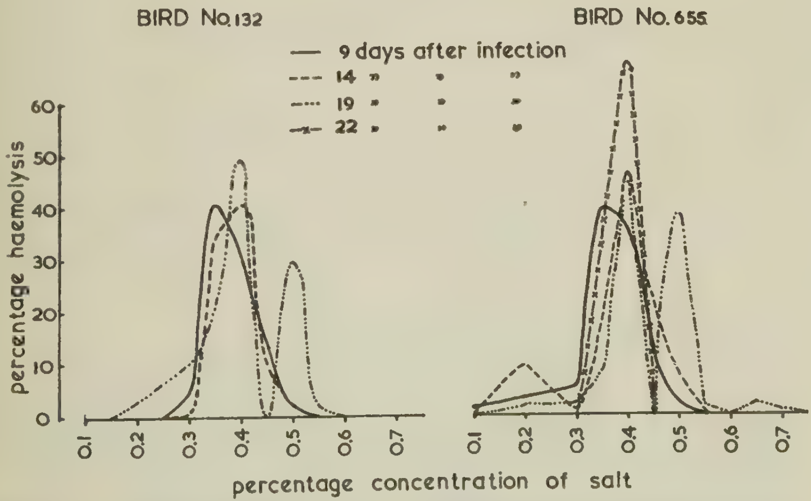


Fig. 10



Figs. 9–10. Showing variations in osmotic fragility patterns of erythrocytes from 3 chickens (Nos. 148, 132 and 655) after oral infection.

Studies of mammalian erythrocytes during anaemic episodes of different aetiologies show certain characteristic features. For example, Suess *et al.* (1948) have observed that during haemolytic anaemias the peak of single curves recording erythrocyte fragility show a 'shift to the right' (i.e. approximately 50 per cent. haemolysis occurring at a higher concentration of salt). A similar shift occurred on the 14th day and subsequent days with erythrocytes from Birds 132 and 655. This may be accounted for either (i) by an alteration in the shape of erythrocytes occurring at this stage of infection, which results in them becoming more spheroidal; (ii) by a slight increase in the CO_2 content of erythrocytes; or (iii) by a modification of erythrocyte surfaces resulting from the release of some product or products of bacterial multiplication in the tissues.

The characteristic binodal curve has been ascribed by Suess *et al.* (1948) to the presence of reticulocytes or target cells which are relatively thin and show maximum lysis in weaker concentrations of salt than those required to lyse the majority of normal erythrocytes. Although Bolton (1949) agrees that a binodal curve reflects the presence of two cell populations of different susceptibilities to NaCl he suggests that reticulocytes alone may not be responsible for this binodal curve.

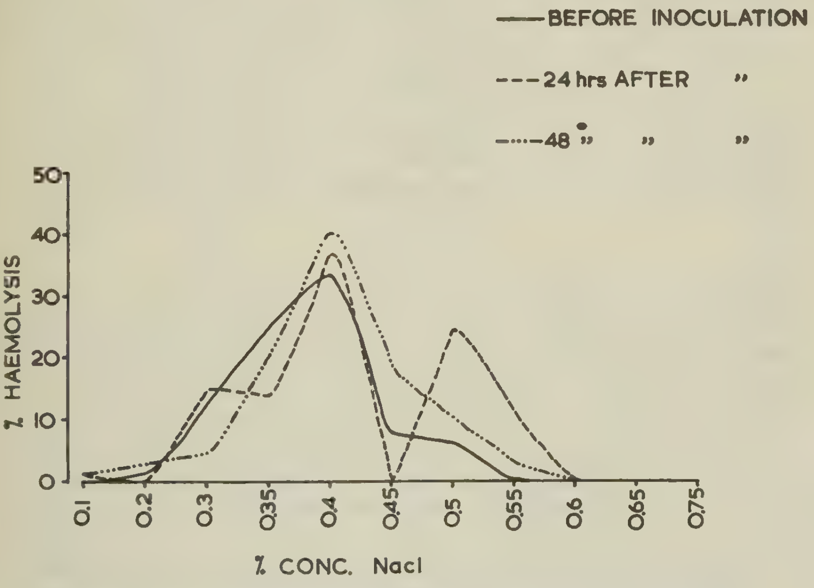
In the experiments recorded above, the second curve of abnormal lysis occurred in all cases at a 0·5 per cent. concentration of salt and showed a marked uniformity in the three birds. Lysis of erythrocytes in this relatively high concentration of salt solution suggests that a considerable proportion of cells are either more spheroidal or have an altered permeability of cell membranes. Moreover, the complete disappearance of this abnormal curve 3 days later in the bird which survived, and the death of the other two birds soon after the appearance of this abnormal fragility, suggests that it is associated with a critical stage of infection.

After intravenous inoculation of Salm. gallinarum *polysaccharide.* It has been shown Buxton (1959) that during a generalised and severe infection with *Salm. gallinarum,* some bacterial polysaccharide becomes adsorbed on to the surfaces of erythrocytes. The previous experiment on erythrocyte fragility indicates that during infection the characteristic binodal curve can be recorded for a short time prior to death of a chicken and that it soon disappears in a bird which survives. Thus, this phase of abnormal fragility is closely associated with the acute stage of infection.

The purpose of this experiment was to observe whether the intravenous inoculation of *Salm. gallinarum* polysaccharide into a normal chicken would result in a similar binodal curve representing abnormal erythrocyte fragility. Fig. 11 records the typical picture of erythrocyte fragility at 24 and 48 hours after intravenous inoculation of 2 doses each of 1·0 ml. polysaccharide at an interval of 30 minutes. On the day after inoculation, the fragility test gave a distinct binodal curve with the two peaks in similar positions to those recorded previously for infected birds. On the second day following inoculation the erythrocyte fragility had returned to practically normal, showing a slight tail extending to 0·6 per cent. concentration of salt.

To produce this distinct binodal curve of fragility it was found necessary to inoculate relatively large quantities of polysaccharide intravenously, similar to the two doses required to temporarily saturate the reticulo-endothelial system for the demonstration of in vivo sensitisation of erythrocytes with polysaccharide (Buxton, 1959). Haemoglobin estimations on birds which received bacterial polysaccharide intravenously showed no abnormal variation during the ensuing 48 hours, and packed cell volumes also remained constant. Moreover, the temporary nature of the binodal curve under these circumstances suggests that the abnormal fragility may have resulted from a temporary alteration in either the shape of erythrocytes or the permeability of their membranes consequent upon intravenous inoculation of polysaccharide.

Fig. 11

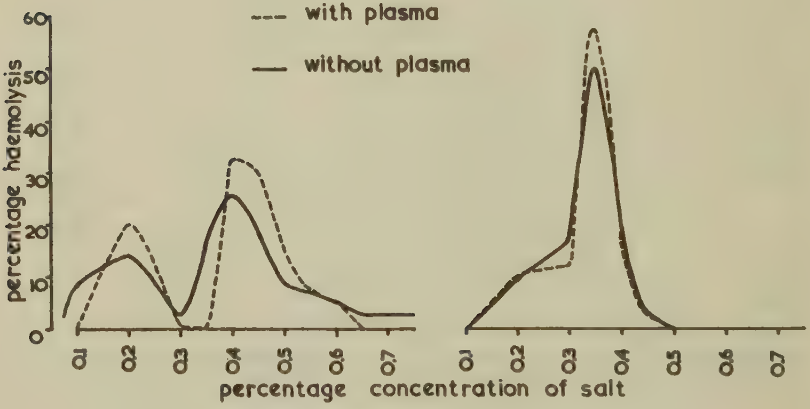


Showing typical variations in osmotic fragility patterns of erythrocytes from a chicken following intravenous inoculation of *Salm. gallinarum* polysaccharide.

After sensitisation with Salm. gallinarum *polysaccharide.* A marked similarity has been observed between the fragility of erythrocytes in chickens during the critical stage of infection with *Salm. gallinarum* and in normal birds soon after intravenous inoculation of *Salm. gallinarum* polysaccharide. Under both conditions two cell populations develop which show distinct osmotic fragility patterns. The relationship of these patterns to bacterial polysaccharide and infection with *Salm. gallinarum* suggests that they result from the adsorption of bacterial polysaccharide on to the surfaces of erythrocytes. The following experiment was designed to show whether a similar binodal curve showing two cell populations of distinct osmotic fragility could be demonstrated in vitro by adsorbing polysaccharide on to erythrocytes.

A sample of heparinised whole blood was obtained from a healthy adult chicken and the plasma removed and stored at 5°C. until required. The erythrocytes were washed three times in saline (pH 7·2) and divided into two equal portions. To one portion was added neutralised bacterial polysaccharide and to the other a similar volume of saline. Each sample was then incubated in a water bath at 37°C. for 1 hour. Subsequently, each sample of erythrocytes was washed three times in saline and divided into equal portions. One sample of sensitised cells and one sample of control cells were resuspended in their original volumes of plasma, and the remaining two samples were suspended in similar volumes of saline. All four samples of erythrocytes were then tested for osmotic fragility and the results are recorded in Fig. 12.

Fig. 12



SENSITISED ERYTHROCYTES CONTROL ERYTHROCYTES

Showing changes in osmotic fragility of avian erythrocytes after sensitisation in vitro with *Salm. gallinarum* polysaccharide.

The fragility patterns of normal, washed erythrocytes in saline and in homologous plasma were similar and showed a single curve with a peak at 0·35 per cent. concentration of salt, and a distinct tail at 0·2 per cent. salt. Sensitised erythrocytes showed a distinct binodal curve with increased haemolysis at 0·2 per cent. saline and the recordings for sensitised erythrocytes in plasma are similar to those for erythrocytes from Bird 655 on the 14th day after infection

(Fig. 10). In this experiment the haemolysis of sensitised cells over an increased range of salt concentrations in a distinct binodal pattern occurred under conditions where cellular changes resulting from anaemia do not exist. Moreover, this abnormal pattern of osmotic fragility, resulting from sensitisation of a sample of erythrocytes from a normal chicken, indicates that the binodal curve results from either uneven sensitisation of cells or from variations in osmotic fragility, possibly due to shape of erythrocytes, which may be enhanced by adsorption of polysaccharide. The position of the curves in relation to concentrations of salt solution from this in vitro experiment cannot be compared too rigidly with the position of similar curves from in vivo experiments because of the preliminary handling of the erythrocytes in the former experiment. Dacie and Vaughan (1938), working with mammalian erythrocytes, have demonstrated how various physical factors can alter osmotic fragility. For example, cell volumes increase at pH 7·2 compared with pH 8·1 (Parpart *et al.*, 1947). Variations in O_2 and CO_2 content of blood markedly alter fragility due to the increased size of erythrocytes in which the proportion of CO_2 becomes augmented (Whitby and Hynes, 1935; Creed, 1938), and Dacie and Vaughan (1938), have emphasised the alteration of fragility patterns of erythrocytes which have been previously washed. Finally, sensitisation of erythrocytes in vitro is maximal insofar as subsequent serological tests are concerned, whereas, in vivo sensitisation is only partial.

DISCUSSION

Heterologous erythrocytes after inoculation into an animal are removed within a few minutes from the circulation by the liver and spleen (Cutbush and Mollison, 1958). Under favourable conditions in which the reticulo-endothelial system is not already temporarily saturated with bacterial polysaccharide, the inoculation of homologous erythrocytes, previously sensitised with bacterial polysaccharide, will result in their rapid removal from the circulation. This has been confirmed in a number of experiments in which volumes of 20 to 25 ml. blood have been withdrawn from normal chickens, the erythrocytes sensitised to a maximum degree with polysaccharide and then re-inoculated into the original birds. No trace of these sensitised erythrocytes could be detected 30 minutes after their re-inoculation by using any of the following tests; an antiglobulin haemagglutination test; a double antiglobulin haemagglutination test incorporating rabbit anti-avian globulin serum followed by absorbed sheep anti-rabbit globulin serum; and a test similar to that of Jones and Silver (1958) in which normal avian erythrocytes, previously sensitised with polysaccharide and homologuous antibody, were added to the test erythrocytes after the latter had been subjected to the antiglobulin haemagglutination test.

The severe haemolytic anaemia which develops characteristically in the subacute form of fowl typhoid may be the result of one or other of several immunological reactions. The in vivo sensitisation of erythrocytes with bacterial polysaccharide results in their rapid removal from the circulation by the reticulo-endothelial system, and antigen-antibody reactions may occur on the surfaces of erythrocytes between bacterial polysaccharide and *Salm. gallinarum* antibody. On a number of occasions erythrocytes have been withdrawn from birds in the acute phase of the disease, washed and mixed with rabbit anti-avian globulin serum. Such treatment has resulted in the haemagglutination of these erythrocytes provided that the latter were obtained shortly before death of the bird; namely, under conditions when multiplication of *Salm. gallinarum* was generalised and rapid, and when serum antibody titres were high. The relationship between antigen-antibody reactions and circulating erythrocytes is the subject of further investigation. The demonstration of positive antiglobulin haemagglutination tests only during the acute stage of disease, and the experiments of Jandl and Tomlinson (1958) on the host's reaction to homologous and heterologous erythrocytes and haemoglobin, is evidence in favour of a specific antigen-antibody reaction occurring in place of a non-specific adsorption of plasma protein on to erythrocytes.

In other animal species it has been shown that haemagglutination arising from antigen-antibody reactions results in the removal of erythrocytes by the liver, lungs and spleen (Jandl, Jones and Castle, 1957; Mollison and Hughes Jones, 1958). It is also possible that the phenomenon of immune adherence described by Nelson (1953, 1956) and Turk (1958) may be concerned with the destruction of erythrocytes. The demonstration of these immune reactions in vivo is handicapped by the rapid removal of polysaccharide by the reticulo-endothelial system and by the fact that saturation of this system with polysaccharide may result in the sudden death of the host. Even before saturation has been reached, accumulation of polysaccharide (e.g. *Salm. typhosum*) by cells of the reticulo-endothelial system may lead to the concentration of leucocytic pyrogen in the blood and a rise in body temperature (Grant and Whalen, 1953; Atkins and Wood, 1955).

Evidence that some of the foregoing immune phenomena may occur in a severely infected chicken is reflected in the experimental results demonstrating the development of an haemolytic anaemia. It has been indicated that erythrocytes which have become sensitised in vivo with bacterial polysaccharide are removed from the circulation and then destroyed by the reticulo-endothelial system. Similarly, if an antigen-antibody reaction takes place on the surfaces of erythrocytes these cells will be similarly removed from the circulation and destroyed. Thus, in both these circumstances the anaemia which develops would not be the result of intravascular haemolysis. This has been confirmed by many tests for the presence of plasma haemoglobin, using Dacie's (1956) modification of the method described by Bing and Baker (1931) which have failed to show any significant increase in the levels of haemoglobin in plasma.

The occurrence of abnormalities of erythrocytes with other salmonella infections in man and animals has not been generally stressed but there are several recorded observations on this feature of these diseases. For example, typhoid fever in man may be accompanied by an acute haemolytic anaemia (Flandin, Bernard and Mallarmé, 1935; Berman, Braun and Rachmilewitz, 1945) and haemoglobinuria (Shaw, 1951); and there have been suggestions that disorders of the blood may result in a definite predisposition to salmonella infection (Storey, 1958). Evidence of abnormalities of erythrocytes associated with *Salm. gallinarum* infection is provided by the experimental results on alterations in osmotic fragility of erythrocytes during infection. The reflection of this in a binodal curve under varying experimental conditions has shown that abnormal fragility may result from the sensitisation of erythrocytes with polysaccharide rather than from the presence of immature cells in the circulation, and these sensitised cells are removed rapidly by the reticulo-endothelial system.

Ho and Kass (1958) have observed that after intravenous inoculation of *Salm. typhi* endotoxin into normal rabbits a haemolytic anaemia developed. A more marked anaemia developed in immunized rabbits which were subjected to repeated inoculations of endotoxin – the haemoglobin level falling by 8 to 12 g. per cent. During this time erythrocyte fragility remained unchanged. These findings confirm experimental results described in this paper since alteration of erythrocyte fragility occurred only for a relatively short period of time after intravenous inoculation of large doses of *Salm. gallinarum* polysaccharide into chickens. Moreover, in infected chickens, abnormal fragility of erythrocytes was demonstrated only when the peak of bacterial infection had developed. The suggestion by Ho and Kass that the development of an haemolytic anaemia is the result of increased activity of the reticulo-endothelial system is in agreement with our own conclusions that during the development of *Salm. gallinarum* infection the reticulo-endothelial system becomes stimulated and is able increasingly to take up liberated bacterial polysaccharide, including erythrocytes which have become sensitised with polysaccharide.

CONCLUSIONS

Chickens fatally infected with fowl typhoid may develop a severe haemolytic anaemia. During the three days preceding death, haemoglobin concentrations showed a rapid decline to approximately one half or one quarter of the normal levels of 12 to 17 g. per 100 ml. Chickens which survived oral dosage with *Salm. gallinarum* showed reduced haemoglobin levels on the 8th day after infection, and these levels began to return to normal on the 10th to 14th days in those birds which became clinically normal. However, in chickens which died a few weeks later, haemoglobin levels remained at a constant level of 6 to 8 g. per 100 ml.

During the acute stage of infection, circulating erythrocytes developed an abnormal osmotic fragility which was characteristic of a generalised bacterial infection.

These results are discussed in relation to the in vivo adsorption of *Salm. gallinarum* polysaccharide on to erythrocytes and to the responses of other animal species and man to similar endotoxins.

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THE PATHOGENICITY OF *PASTEURELLA HAEMOLYTICA* FOR YOUNG LAMBS

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INTRODUCTION

The name *Past. haemolytica* was introduced by Newsom and Cross (1932) and designates organisms similar to those described as *B. bovisepticus* Group I by Jones (1921). *Past. haemolytica* is associated with disease in sheep and cattle in many parts of the world. Montgomerie, Bosworth and Glover (1938) gave an account of enzootic pneumonia in sheep. Woxholtt, Naerland and Hoff (1952) described an acute disease of lambs between one and two months of age, and deaths in new-born lambs due to *Past. haemolytica* were reported by Hartley and Boyes (1955). Stamp, Watt and Thomlinson (1955) described a septicaemic disease occurring among lambs in the autumn.

Difficulties associated with producing experimental infections in sheep have been chiefly responsible for the doubt in which the pathogenic significance of the organism has frequently been held and the literature contains no conclusive evidence that *Past. haemolytica* can be of high virulence for the ovine species. Montgomerie *et al.* (1938) failed to reproduce enzootic pneumonia and suggested the possible aetiological involvement of a virus. This suggestion was put forward again by Downey (1957) who also referred to a personal communication from Hartley and Boyes to the effect that intravenous or intraperitoneal inoculation of new-born lambs with *Past. haemolytica* resulted in a rapidly fatal infection; older lambs and sheep were said to resist intraperitoneal injections. The septicaemic disease of lambs was reproduced by Stamp *et al.* (1955) and later by Biberstein and Kennedy (1959) but the doses used were large.

Smith (1959) classified each of a number of sheep strains as one of two Types, designated A and T. Cases of enzootic pneumonia yielded A strains while lamb septicaemias as described by Stamp *et al.* (1955) were associated with Type T infections.

Generalised Type A infections have recently been recognised in very young lambs and field observations suggested that these animals might be particularly susceptible to experimental infection with a freshly isolated A strain. Investigation of this point constituted the main purpose of the following series of experiments.

MATERIALS AND METHODS

Strains. Two strains of *Past. haemolytica* were used. Both produced narrow zones of haemolysis on blood agar, gave slight growth on McConkey medium, were indol negative and reduced nitrate to nitrite. Fermentation reactions were carried out in the medium recommended by Bosworth and Lovell (1944). Positive reactions were given in xylose, glucose, fructose, mannose, sucrose, maltose, dextrin, mannitol and sorbitol. Gas was not produced. Inulin, erythritol, adonitol and dulcitol were not fermented. Virulence for mice was low. Each strain was typed using the methods outlined previously by Smith (1959).

Strain 158 was a Type A organism obtained from a 3 week old lamb which died at the Moredun Institute during the 1959 outbreak (see Field Observations). At post-mortem the only macroscopic abnormalities in this lamb consisted of a few sub-pleural haemorrhages on the chest wall and numerous subscapsular, pin-point, whitish foci in the liver. Histologically the liver and lungs showed many scattered foci of bacteria. Using the method of counting described below the liver and lungs were shown to contain at the time of death 750×10^6 and 207×10^6 viable organisms per gramme respectively.

Strain 7157H was a Type T organism isolated in 1954 from septicaemia in a 6 month old lamb.

Experimental animals. Three to 4 week old lambs were obtained from two farms. The adult sheep used consisted of 1 and 2 year old animals from the Institute flock. Mice were approximately 4 weeks old at the time of inoculation.

Intraperitoneal and intravenous inocula. These consisted of dilutions of 5 hour infusion broth cultures. Immediately before injection viable counts of bacteria in inocula were performed by the method of Miles and Misra (1938). The average of 3 replicate counts was taken to be the true value.

Intratracheal inoculations. Inocula consisted either of 5 hour infusion broth culture or of suspensions of 18 hour blood agar culture in aqueous 1 per cent. casein hydrolysate. Injections were carried out under deep ether anaesthesia.

Blood cultures. 1 ml. volumes of blood were withdrawn aseptically from the jugular vein and inoculated into 10 ml. of broth. After incubation these cultures were examined for *Past. haemolytica.*

Counts of viable bacteria in tissues. Immediately or shortly after death autopsies were carried out and portions of various tissues were transferred to the refrigerator. Within a few hours thereafter weighed amounts of each organ were removed aseptically. The amount of tissue taken was usually 5 g. but occasionally only 1 g. could be conveniently obtained. The method consisted of immersing a large piece of tissue for 5 to 10 seconds in boiling water and subsequently dissecting out the required weight from the central, unheated part using sterile instruments. The tissue was then finely chopped with scissors, diluted in 1 per cent. casein hydrolysate solution to make a 1/5 W/V suspension and finally macerated in a Griffith tube. Serial ten-fold dilutions were prepared from each resulting suspension and 0·02 ml. volumes from each dilution were transferred to the surface of blood agar by means of a calibrated dropping pipette. Colonies were counted after 18 hours' incubation. No replicate counts were performed. Fluids such as peritoneal exudates were collected aseptically and dilution series for counting were prepared directly from them.

RESULTS

Field Observations

In the spring of 1959 a number of deaths in very young lambs was investigated and found to be associated with generalised infection of *Past. haemolytica.* The lambs involved varied from 2 days to 12 weeks in age but the majority were 3 to 4 weeks old. Twelve cases occurred in the Moredun Institute flock and in addition 6 cases from other sources were examined. The majority showed fibrinous pleurisy with adhesions to the chest wall and small areas of pneumonia were usually associated with these lesions. Pericarditis was sometimes present and several lambs showed sub-epicardial, pericardial and sub-pleural haemorrhages, the latter occurring beneath both visceral and parietal pleura. One lamb showed acute fibrinous peritonitis in addition to thoracic lesions. In 2 cases acute fibrinous peritonitis with adhesions and turbid exudate was the only visible abnormality. The liver of one lamb showed numerous subcapsular, pin-point, whitish foci.

On surface culture pneumonic lung tissue invariably yielded heavy confluent growth of *Past. haemolytica*. Slightly less luxuriant but still very considerable growth was obtained from apparently normal lung tissue, liver and spleen. Kidney cultures were always positive but the numbers of bacteria present were often much smaller. The organism could be regularly isolated from the tracheal mucous membrane.

Of the 18 cases examined 17 yielded *Past. haemolytica* Type A and only 1 yielded Type T.

Preliminary Pathogenicity Test using Strain 158 (Type A)

Two lambs were inoculated with the freshly isolated Strain 158, one intraperitoneally and the other intravenously. A primary culture on blood agar was used to inoculate infusion broth which was then incubated for 5 hours. The dose of broth culture per lamb was 5 ml. of a 1/50 dilution and this was shown to contain $11{\cdot}5 \times 10^6$ viable bacteria.

Lamb 1. This animal was inoculated intraperitoneally and it died after 12 hours from an acute, fibrinous peritonitis. The abdominal cavity contained about 30 ml. of pale amber, turbid fluid. The diaphragmatic surface of the liver was encased in fibrin. The intestines were congested. The thorax contained approximately 20 ml. of slightly turbid, amber fluid and a small number of sub-pleural haemorrhages was present on the chest wall. The lungs were apparently normal except for a few small sub-pleural, haemorrhagic spots and several tiny areas of collapse. Counts of viable bacteria in tissues and fluids gave the following results:

Lung, $0{\cdot}5 \times 10^6$ per g.; liver, $0{\cdot}1 \times 10^6$; spleen, $0{\cdot}2 \times 10^6$; kidney, $3{\cdot}0 \times 10^6$; peritoneal exudate, 18000.0×10^6 per ml.; pleural, exudate, $205{\cdot}0 \times 10^6$.

Lamb 2. The inoculum was given intravenously into the left jugular vein. Next day the lamb was acutely lame on the right foreleg. Its temperature was 106·9°F. and a blood culture demonstrated the presence of circulating *Past. haemolytica*. The following day the right foreleg was extremely swollen and painful and the animal showed a temperature of 106·2°. It was slaughtered on humane grounds. On post-mortem the only abnormality seen was in the affected foreleg in which a severe purulent arthritis was present in the knee and fetlock. The copious greenish-white pus yielded a pure culture of *Past. haemolytica*. Lung, liver and kidney were bacteriologically sterile and a plug of spleen tissue taken with a Pasteur pipette yielded only one colony on culture.

Intraperitoneal Inoculation of Young Lambs and Adult Sheep with Graded Doses

Strain 158 (*Type A*). A primary blood agar culture of Strain 158 derived from Lamb 1 was used to inoculate infusion broth which after 5 hours incubation contained 290×10^6 viable organisms per ml. The various dose levels used were prepared from this broth culture. Pairs of lambs were inoculated with doses of 58×10^6, 58×10^4, 58×10^2 and 58 viable bacteria contained in dose volumes of 2·0 ml. Pairs of adults were inoculated in the same way except that the smallest dose was omitted. Each pair of adults consisted of a 1 year old and a 2 year old sheep.

The results are shown in Table 1. All deaths occurred within 24 hours of inoculation. The survivors were kept for 7 days before being discarded. It is clear that the organism was of high virulence for the lambs, amongst which 6 deaths occurred. Prior to death, elevated temperatures ranging from 105·3° to 108°F. were observed. The adult sheep were completely resistant to the doses used and no elevated temperatures, symptoms or deaths occurred in these animals. The post-mortem appearances of the dead lambs were essentially similar to that of Lamb 1. Acute, fibrinous peritonitis was present and the quantities of peritoneal exudate varied from approximately 10 ml. to 100 ml. The volumes of pleural exudate ranged from 5 ml. to 20 ml. Counts of viable bacteria in various tissues and exudates were carried out and are recorded in Table 2. Peritoneal fluids contained enormous numbers of organisms and it is clear that multiplication in vivo occurred to a very high degree.

Strain 7157H (*Type T*). Strain 7157H had been maintained for a considerable period in the laboratory. The experimental design and methods were precisely similar to those used in the previous experiment. In this case however the 4 dose levels contained 140×10^6, 140×10^4, 140×10^2 and 140 viable organisms. Only one death was produced and this in a lamb which received the largest dose. It died with acute, fibrinous peritonitis 12 hours after inoculation. One lamb which had received 140×10^4 organisms showed a temperature of 105·3° next day but this rapidly subsided. None of the other lambs or adults showed a temperature rise or symptoms. After 7 days survivors were discarded. The results of viable counts of organisms in tissues and exudates of the dead lamb were as follows:

Lung, 57×10^6 per g.; liver, $0{\cdot}07 \times 10^6$; spleen, $0{\cdot}2 \times 10^6$;

peritoneal exudate (15 ml. present), 8,500 × 10^6 per ml.; pleural exudate (20 ml. present), 900 × 10^6.

TABLE 1

INTRAPERITONEAL VIRULENCE TITRATIONS OF STRAIN 158 IN YOUNG LAMBS AND ADULT SHEEP

Dose of viable organisms	*Deaths*	
	Lambs	*Adults*
58 × 10^6	2/2	0/2
58 × 10^4	1/2	0/2
58 × 10^2	2/2	0/2
58	1/2	N

N = Not done

TABLE 2

BACTERIOLOGICAL EXAMINATION OF 6 LAMBS WHICH DIED AFTER INTRAPERITONEAL INJECTIONS OF STRAIN 158

Doses of viable bacteria received by 6 lambs (millions)	*Viable counts of bacteria in tissues and exudates (millions per g. or ml.)*			
	Lung	*Liver*	*Peritoneal exudate*	*Pleural exudate*
58·0	1·0	0·007	18,500·0	26·0
58·0	375·0	5·0	45,000·0	17,000.0
0·58	4·0	0·1	27,500·0	3,500·0
0·0058	12·0	4·0	45,000·0	150·0
0·0058	N	N	15,500·0	N
0·000058	N	N	2,350·0	N

N = Not done

Intravenous Inoculation of Young Lambs, Adult Sheep and Mice with Graded Doses of Strain 158

Pooled peritoneal exudates from the lambs whose deaths are recorded in Table 1 were cultured on blood agar. A single colony was then inoculated into infusion broth which after 5 hours incubation contained 670 × 10^6 viable organisms per ml. This broth culture was used to prepare various doses for animal inoculation. Four dose levels were used and these consisted of 335 × 10^6, 335 × 10^4, 335 × 10^2 and 335 living bacteria. The smallest dose was tested intravenously in a group of 3 lambs. The larger doses were each tested intravenously in groups of 3 lambs, 2 adult sheep and 6 mice, and intraperitoneally in a group of 6 mice. The dose volumes used were 5·0 ml. for lambs and sheep and 0·5 ml. for mice.

TABLE 3

VIRULENCE TITRATIONS OF STRAIN 158 IN LAMBS, ADULT SHEEP AND MICE

Dose of viable bacteria	*Deaths and survivals*			
	Inoculated intravenously			*Inoculated intra-peritoneally*
	Lambs	*Adult sheep*	*Mice*	*Mice*
335 × 10^6	Lamb No. 11 D „ „ 12 D „ „ 13 D	2/2 S	6/6 S	6/6 S
335 × 10^4	Lamb No. 14 D „ „ 15 D „ „ 16 D	2/2 S	6/6 S	6/6 S
335 × 10^2	Lamb No. 17 S „ „ 18 S „ „ 19 S	2/2 S	6/6 S	6/6 S
335	Lamb No. 20 D „ „ 21 S „ „ 22 S	N	N	N

D = Died
S = Survived
N = Not done
Lambs No. 11, 12 and 13 died within 24 hours of inoculation.
Lambs No. 14, 15, 16 and 20 died 8, 16, 7 and 13 days respectively after inoculation.
Lambs No. 14, 15 and 20 yielded *Actinobacillus lignieresi* in addition to *Past. haemolytica* on culture.

Table 3 shows the fates of the inoculated animals. Seven lambs died, but all adult sheep and mice were resistant to the doses used. On the day after inoculation the adults showed normal temperatures and blood cultures were negative. Mice which received the largest dose level intraperitoneally lost weight and showed signs of illness in the following 48 hours but they rapidly recovered. In all other mice the inocula produced no observable effect. Three of the 7 lambs which died were animals which had received the largest dose level and they succumbed within 24 hours of inoculation. The four other deaths occurred in lambs receiving smaller doses and were delayed for periods ranging from 8 to 16 days after inoculation. The surviving lambs were slaughtered and examined after 22 days at which time they showed no outward signs of illness. Blood cultures were performed at intervals throughout the experiment and the results which are recorded in Table 4 show that circulating bacteria could be demonstrated at times in the majority of lambs. Many of the lambs destined to die showed fluctuating temperatures which were frequently normal. Counts of viable bacteria in the tissues and exudates of lambs which died and of those which were slaughtered are shown in Table 5. It will be seen that 3 of the lambs, which died, yielded *Actinobacillus lignieresi* in addition to *Past. haemolytica* on culture. Post-mortem observations and in some cases clinical data concerning individual lambs are given below. The lambs are identified as in Table 3 from which information regarding size of infecting dose may be obtained.

TABLE 4

BLOOD CULTURES IN LAMBS INOCULATED INTRAVENOUSLY WITH STRAIN 158

Lamb Nos.	*Blood cultures at intervals after inoculation in days*							
	2	*3*	*6*	*7*	*9*	*10*	*13*	*20*
11	D	D	D	D	D	D	D	D
12	D	D	D	D	D	D	D	D
13	D	D	D	D	D	D	D	D
14	+	+	—	—	D	D	D	D
15	+	—	—	—	—	—	—	D
16	+	+	+	D	D	D	D	D
17	—	—	—	—	—	—	—	—
18	—	—	—	—	—	—	—	—
19	—	—	—	+	+	—	+	—
20	—	—	—	—	—	—	+	D
21	+	—	—	—	—	—	—	—
22	+	—	—	—	—	—	—	—

+ = *Past. haemolytica* present.
— = „ „ absent.
D = Lamb dead.
Each culture consisted of 1·0 ml. blood in 10·0 ml. broth.
Inocula are described in Table 3.

Lambs 11, 12 and 13. Death from acute, fibrinous peritonitis occurred within 24 hours of inoculation. The post-mortem appearance of each lamb was essentially similar to that of Lamb 1 which has already been described. Volumes of peritoneal exudate varied from approximately 30 ml. to 60 ml. Two of the three lambs showed petechiation of the abomasal mucosa. Enormous numbers of bacteria were present in peritoneal exudates.

Lamb 14. Death took place 8 days after inoculation. Pleurisy with adhesions but only a trace of exudate was present. The apical lobe of the right lung showed areas of consolidation and was covered with a layer of fibrin. Both lungs were generally congested and purplish in colour with a number of sub-pleural haemorrhages. The pericardium was thickened and covered with petechiae. Haemorrhages were also present under the epicardium, on the thoracic surface of the diaphragm and under the parietal peritoneum. There was marked peritonitis with fibrin, adhesions and about 10 ml. of cloudy exudate. This exudate contained *Past. haemolytica* in profuse numbers. *Actinobacillus lignieresi* was also isolated from the carcase.

TABLE 5

BACTERIOLOGICAL EXAMINATION OF LAMBS WHICH DIED OR WERE SLAUGHTERED AFTER INTRAVENOUS INJECTIONS OF STRAIN 158

Lamb Nos.	*Viable counts of* Past. haemolytica *in tissues and exudates (millions per g. or ml.)*					
	Lung	*Liver*	*Spleen*	*Kidney*	*Peritoneal exudate*	*Pleural exudate*
11	0·9	0·05	6·2	0·2	12,000·0	285·0
12	102·0	10·0	65·0	2·5	20,000·0	N
13	0·07	0·4	12·5	0·2	6,500·0	7,500·0
14	F*	F*	0·07	F*	21,500·0	N
15	550·0	1·2*	67·0	N	0*	0*
16	1100·0	1075.0	6·0	0	0·03	1·2
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	1·1	0	0	0	0·7	N
20	250·0	10·2	4·2	N	0*	N
21	0	0	0	0	0	0
22	0	0	0	0	0	0

Inocula and fate of lambs described in Table 3.

Where pulmonary lesions present viable counts were performed on abnormal lung tissue.

F = *Past haemolytica* present, but less than 0·02 × 10^6 per g. of tissue.

* = *Actinobacillus lignieresi* present.

N = Not done.

Lamb 15. On the day following inoculation this animal showed lameness which became progressively worse until after 14 days both hind legs were virtually paralysed. Severe respiratory symptoms appeared shortly before death which occurred 16 days after inoculation. On post-mortem marked fibrinous pleurisy with adhesions was seen. A considerable amount of greenish cloudy pleural exudate was present. The lungs were slate-blue in colour and showed sub-pleural haemorrhages but there were no areas of consolidation. The pericardial sac contained a small amount of fibrinous exudate. There was a fibrinous peritonitis with adhesions and several hundred millilitres of cloudy, greenish fluid. A spinal abscess several centimetres in diameter was found in the lumbar region and this yielded a mixture of *Past. haemolytica* and *Actinobacillus lignieresi* on culture. The latter organism was isolated from several other sites. Lung and spleen tissue yielded large numbers of *Past. haemolytica.*

Lamb 16. Slight lameness was shown on the day following inoculation and persisted until death which occurred after 7 days. At post-mortem subcutaneous blood vessels were seen to be injected. Fibrinous pleurisy with adhesions and about 15 ml. of blood-tinged pleural exudate was present. The right lung was covered with fibrin which was easily stripped off to show surface haemorrhages and generalised congestion of lung tissue. Consolidation and collapse were present in the apical lobe only. The left lung was pink and normal in appearance. Fibrinous pericarditis with a considerable amount of exudate and sub-epicardial haemorrhages were observed. There was a fibrinous peritonitis with adhesions and about 60 ml. of clearish fluid. Very large numbers of pin-point, whitish foci were present on the surface and in the substance of the liver. These lesions were similar to those present in the original lamb from which the experimental strain of *Past. haemolytica* was isolated. The surface of the liver showed many haemorrhages. The intestines were inflamed. No lesions were visible in joints. An abscess found in the cortex of one kidney yielded *Past. haemolytica.* Very large numbers of this organism were present in tissue removed from both consolidated and non-consolidated areas of the right lung and from the liver.

Lamb 17. This animal was slaughtered 22 days after inoculation. The only abnormality observed was the presence of about 10 ml. of clearish peritoneal fluid. The carcase was bacteriologically sterile.

Lamb 18. When slaughtered 22 days after inoculation the only visible abnormality consisted of a number of whitish foci, 0·25 cm. in diameter, on the surface and in the substance of the liver. These and other parts of the carcase were bacteriologically sterile.

Lamb 19. This lamb was also slaughtered 22 days after inoculation. The left lung showed fibrinous pleurisy with adhesions and an underlying pneumonia affecting the apical and cardiac lobes and part of the diaphragmatic lobe. In the abdominal cavity a few strands of fibrin were present together with about 10 ml. of cloudy fluid. A considerable number of haemorrhages under the parietal peritoneum were observed. Pneumonic lung tissue and peritoneal exudate contained relatively small numbers of *Past. haemolytica.* Other sites cultured were bacteriologically sterile.

Lamb 20. Death occurred 13 days after inoculation. Symptoms of lameness had been observed. A thick, extensive layer of fibrin was present on the left lung and there were adhesions to the chest wall.

After removal of the fibrin the lung was seen to be congested and covered with numerous small haemorrhagic areas. Many tiny, whitish foci were scattered throughout the tissue of both lungs. There was a severe peritonitis, the intestinal folds being firmly stuck together with rubbery material. Approximately 5 to 10 ml. of cloudy fluid were present in the abdominal cavity. The liver was completely encased in fibrin and a number of whitish lesions up to 0·5 cm. in diameter were seen on the surface and in the substance of the organ. The abomasal mucosa showed several large haemorrhagic areas. The joints were apparently normal. *Past. haemolytica* was isolated from several sites and large numbers were present in lung tissue. *Actinobacillus lignieresi* was cultured from peritoneal exudate.

Lamb 21. The animal was slaughtered 22 days after inoculation. On post-mortem the only abnormalities consisted of about 5 ml. of cloudy peritoneal fluid and several lesions on the surface of the liver which took the form of small abscesses up to 1 cm. in diameter. These were frequently surrounded by a zone of tiny, haemorrhagic spots. The carcase was bacteriologically sterile.

Lamb 22. As for Lamb 17.

Morphological and cultural characteristics together with reactions in the presence of 24 fermentable substances were determined for cultures of *Past. haemolytica* isolated from the carcases of Lambs 14, 19 and 20. The properties were in each case found to be those of the strain inoculated.

Intratracheal Inoculation of Young Lambs with Strain 158

Lambs No. 23, 24 and 25 were inoculated intratracheally. Lamb 23 received 1 ml. of the 5 hour broth culture used in the previous experiment and this dose contained 670 × 10^6 viable organisms. The same broth culture was used as inoculum for a blood agar culture from which, after 18 hours incubation, a suspension of *Past. haemolytica* in 1 per cent. casein hydrolysate solution was prepared. This suspension contained 13,000 × 10^6 viable bacteria per ml. and was administered to Lambs 24 and 25 in doses of 1 ml. and 2 ml. respectively.

Lamb 23. Following inoculation this animal showed no symptoms of ill-health and blood cultures were consistently negative. After 22 days the lamb was slaughtered and on post-mortem examination the apical and cardiac lobes of the right lung were found to be pneumonic. The lesion which in extent was similar to that frequently seen in enzootic pneumonia was pale red in colour and appeared to be resolving. Pneumonic lung tissue contained the relatively small number of 0·85 × 10^6 viable organisms per gram. Liver, spleen and kidney were bacteriologically sterile.

Lamb 24. A temperature of 106·2°F. was observed and a blood culture was positive on the day after inoculation. After a further 24 hours the temperature had become normal. Several of the blood cultures performed subsequently proved to be positive and the lamb died 7 days after inoculation. On post-mortem lesions of enzootic pneumonia were seen. The apical and cardiac lobes of the right lung were deep red, consolidated and covered with a thick layer of fibrin. The remainder of the right lung was slightly congested but the left lung appeared normal. Adhesions were present in the chest but there was little pleural exudate.

A fibrinous pericarditis was observed. In the abominal cavity there was a fibrinous peritonitis with adhesions and about 25 ml. of cloudy exudate. Counts of *Past. haemolytica* in various tissues and exudates gave the results shown below.

Apical lobe of right lung, 80 × 10^6 per g; diaphragmatic lobe of left lung, 165 × 10^6; liver, 55 × 10^6; spleen, 375 × 10^6; kidney, 0·07 × 10^6; peritoneal exudate, 5,500 × 10^6 per ml.

Lamb 25. Respiratory symptoms were apparent 24 hours after inoculation and the lamb showed a temperature of 106·9°. Death took place on the second day and prior to this blood culture had demonstrated the presence of circulating organisms. Post-mortem examination showed severe pleurisy with many adhesions and several hundred millilitres of exudate. The left lung was entirely covered with fibrin. Both lungs showed a generalised, livid red colour and interlobular oedema. Small dark red areas of consolidation were scattered over lung surfaces and both diaphragmatic lobes exhibited circumscribed lesions of pneumonia. The mediastinal tissues were swollen with gelatinous oedema and there was a fibrinous pericarditis with a considerable amount of exudate. Pericardial and sub-epicardial petechiation was observed. More than 100 ml. of clearish fluid were present in the abdominal cavity but there was no fibrin. Viable counts of *Past. haemolytica* in various tissues and exudates gave the following results.

Lung, 4 × 10^6 per g.; liver, 1 × 10^6; spleen, 12 × 10^6; kidney, 0·1 × 10^6; pleural exudate, 15,000 × 10^6 per ml.; pericardial exudate, 1 × 10^6; peritoneal exudate, 0·6 × 10^6.

Using the methods of examination outlined in the preceding section isolates from Lambs 23 and 24 appeared to be identical to the strain of *Past. haemolytica* inoculated.

DISCUSSION

Tweed and Edington (1930), Stamp *et al.* (1955) and Biberstein and Kennedy (1959) reported the production of fatal septicaemic disease in animals by means of intravenous inoculation of *Past. haemolytica* but a feature common to all these experiments was the use of large infecting doses. Stamp *et al.* (1955), while considering that they had established the causal relationship of the organism to a naturally occurring septicaemic disease of lambs, drew attention to the widespread doubt which exists as to the pathogenic significance of the organism in enzootic pneumonia. Dungal (1931) succeeded in reproducing pneumonia in sheep by intratracheal inoculation of an organism which, though non-haemolytic, was stated by Montgomerie *et al.* (1938) to resemble, on broad grounds, an organism associated with enzootic pneumonia in England and Wales. Dungal's clear-cut results have never been repeated despite numerous subsequent attempts.

The findings described in this paper show conclusively that a freshly isolated Type A strain of *Past. haemolytica* was of very high virulence for 3-week-old lambs. Very small doses proved fatal and exceedingly high degrees of bacterial multiplication were demonstrated. The protracted infections which resulted from the intravenous inoculation of small doses were associated with a variety of lesions including pneumonia and it is interesting to recall that Stamp *et al.* (1955) in dealing with septicaemias of older lambs expressed the view that where pneumonic lesions were present they appeared to be secondary to bacterial thrombi in the pulmonary vessels. Several of the protracted cases were complicated by intercurrent *Actinobacillus lignieresi* infections. The results suggest that lambs were probably more susceptible to intraperitoneal inoculation than to intravenous inoculation. The majority of lesions produced in experimental infections have also been encountered in naturally occurring *Past. haemolytica* infections of young lambs. The resistance of young adult sheep to both intraperitoneal and intravenous inoculation of doses which proved fatal to lambs was striking and since some of the sheep used were only 12 months old it appears that this high degree of immunity develops within the first year of life. Stamp *et al.* (1955), as a result of investigations involving 7 to 9 months old lambs, stated that there was some evidence of increased resistance with age. The present studies also showed that mice were highly resistant to infection with doses to which lambs succumbed. Intratracheal inoculation of lambs with large doses was shown to be capable of producing pneumonia and also generalised infection but the effect of smaller doses injected by this route was not investigated. The much lower virulence for lambs of a Type T strain may have resulted from repeated laboratory subculture of the organism.

CONCLUSIONS

The high susceptibility of 3-week-old lambs and the high resistance of young adult sheep to experimental infection with a Type A strain of *Past. haemolytica* were demonstrated. The lesions produced in lambs were similar to those seen in naturally infected animals of comparable age and marked bacterial multiplication occurred in vivo. Intraperitoneal inoculation of small doses resulted in rapidly fatal peritonitis. Intravenous inoculation gave rise to a similar disease or to protracted infections according to the size of the inoculum. The protracted cases showed a variety of lesions which included pneumonia. Pneumonia and also generalised infection were produced by intratracheal inoculation of larger doses.

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SEPTICAEMIA IN VERVET MONKEYS CAUSED BY *STREPTOCOCCUS PYOGENES**

By

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INTRODUCTION

Epidemics of streptococcal infections in man and in domestic and wild animals are common.

Fortunately, however, the great majority of streptococci of animal origin fall into different serological groups from those in man and are usually only slightly pathogenic to man. Conversely, human strains are usually only slightly pathogenic to animals. Lancefield's group A streptococcus (*Streptococcus pyogenes*), in particular, is the main streptococcal pathogen of man and is only rarely incriminated in diseases of animals. It has occasionally been isolated from cases of mastitis in cattle and metritis in the horse (Swift, 1952), but in these cases there have usually been grounds for suspecting a human source of infection. Spontaneous wound infections with *Str. pyogenes* occasionally occur in animals.

Streptococcal diseases of monkeys are rare, but diseases due to group A streptococcus, namely skin infections, "erysipelas" and septicaemia, do occasionally occur (Swift, 1952). In 1936, Seegal, Heller and Jablonowitz reported the isolation of haemolytic streptococci from the pharynx of twenty-eight normal rhesus monkeys out of a total of forty-nine. Nineteen of the strains were of the human variety and there was no evidence that these organisms were causing disease in the monkeys. Pilot (1937) recovered a haemolytic streptococcus of group A from the blood and organs of an orangutan with a fatal septicaemia. Boisvert (1940) isolated human scarlatinal streptococci from four rhesus monkeys, three of which had spontaneous infections (two with facial erysipelas and one with an axillary abscess) and recovered, while the fourth was accidentally infected and developed a fatal peritonitis.

The object of this paper is to describe the clinical and pathological picture seen during an epidemic of septicaemia due to *Str. pyogenes* in a group of about one hundred vervet monkeys, *Cercopithecus aethiops*, held in captivity in Entebbe, Uganda, and to describe the organism isolated.

RESULTS

The Epidemic

The majority of the monkeys were trapped on the Ssese Islands, Lake Victoria. They were purchased by the Game Department from African trappers and were held at Entebbe until sufficient numbers were collected to warrant shipment overseas. The monkeys were housed in a large outdoor roofed cage which was divided into four separate compartments.

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During the last week of January, 1959, when about one hundred monkeys had been purchased, an outbreak of illness occurred which swept through the colony and reached epidemic proportions. The illness was first noticed as a post partum infection in female monkeys, several of which died before veterinary assistance was requested. The clinical picture reported was acute illness, paralysis of the hind limbs and death. A visit was made to the premises and it was seen that a few of the monkeys showed signs of weakness and diarrhoea; treatment with sulphonamide was prescribed. The next morning, however, a young female monkey was found dead. An autopsy was performed and material was collected for bacteriological and pathological examination. Beta haemolytic streptococci, shown later to belong to Lancefield's group A, were isolated in pure culture from the liver, spleen, kidneys, lungs, myocardium and blood. On the following day two more monkeys were found dead and *Str. pyogenes* was again isolated in pure culture from the organs and blood of both of them and also from throat swabs taken post mortem.

It now became obvious that many of the monkeys were beginning to show signs of illness. It was decided, therefore, to isolate all of the obviously sick ones and to treat each of them initially with 500,000 units of crystalline penicillin followed by daily doses of 500,000 units of procaine penicillin. The remaining monkeys, as well as those in isolation, were fed aureomycin in the form of Aurofac D (Dispersible Aureomycin Chlortetracycline Supplement) at the rate of 1 g./kg. body weight daily, mixed with bananas. This appeared to check the spread of infection since only a few animals died after the treatment was instituted and it is probable that these were already infected. Many of the sick monkeys in isolation recovered. Others died despite treatment with penicillin and *Str. pyogenes* was isolated from four of them; from the liver, spleen and kidneys of three and from the liver only of one. It appeared that even with penicillin and Aurofac treatment, the prognosis for the sick animals was poorer than one would have expected from the apparent degree of illness. The penicillin did not have the spectacular effect on the course of the disease that one had expected, knowing that the causative organism was very sensitive to it. The epidemic lasted about three weeks, during which time approximately thirty monkeys died. Post mortem examinations were performed on fifteen of them and *Str. pyogenes* was isolated from eleven.

The main clinical features of the infection were rapid onset, the monkeys first showing signs of lethargy and weakness which progressed to prostration, and then diarrhoea, fever and fast pulse. In all the later cases facial swellings were seen, causing partial or complete occlusion of the nostrils and eyes and occasionally swelling of the upper lip and under the jowl. *Str. pyogenes* was generally isolated in pure culture from all organs and the blood. A number of the later cases also showed lesions resembling impetigo on the upper eyelids, cheeks and forehead from which *Str. pyogenes* was isolated. Bite wounds of varying severity on the tail and other parts of the body were common. Death usually occurred within forty-eight hours if treatment with penicillin was not instituted at an early stage.

Pathology

At autopsy there was invariably a marked gastro-enteritis, myocarditis and hydropericardium. The liver was always enlarged and soft, and either mottled with small red areas or showing multiple small necrotic foci. The spleen was enlarged and soft. The kidneys were swollen, their cut surfaces bulged and the capsule stripped off easily. The lungs were normal.

From four of the monkeys that died despite treatment with penicillin and Aurofac, no streptococci were isolated. The post mortem picture, however, in these cases was identical to that described above.

Liver. The most marked lesions, as would be expected from the macroscopic examinations, were in this organ; and microscopically three different patterns could be distinguished.

(a) In cases with an acute illness, with isolation of streptococci from all organs, cocci were seen in large numbers in the blood vessels and either in focal areas or throughout the liver parenchyma. Infiltration of mononuclear cells was seen around the blood vessels and a slight degree of venous congestion was evident. Degenerative changes in the hepatic cells were marked, including cloudy swelling, slight fatty degeneration and focal areas of necrosis.

(b) After a sub-acute illness and isolation of streptococci from all organs the most obvious abnormality was severe fatty degeneration of the hepatic cells, with areas of focal necrosis. Small acute abscesses were also seen throughout the parenchyma.

(c) The picture seen after a sub-acute illness and failure to isolate streptococci from any of the organs was again marked fatty degeneration and necrosis of hepatic tissue, either in focal areas or throughout the parenchyma. Congestion of the blood vessels and engorgement of the sinusoids was also evident. No cocci were seen either in the parenchyma or in the blood vessels.

Kidney. In the cortex there was congestion of the larger blood vessels and some haemorrhage. The renal corpuscles were slightly congested but showed no degenerative changes. The tubules showed degenerative changes varying from cloudy swelling to necrosis. There was some infiltration of mononuclear cells around the blood vessels.

Myocardium, lung and spleen. These organs were normal apart from the presence of a few inflammatory cells in the myocardium and some pigment in the spleen.

Lesions on the eyelids. Ulceration was present, with pus and cocci extending laterally from the ulcerated area, partly in the epidermis and partly deep to it. The deeper tissue was diffusely infiltrated with polymorphonuclear neutrophils and many dense accumulations of the same cells were seen in the form of small abscesses. Lymphocytes and plasma cells were also present.

Bacteriology

All isolations were made on ox blood agar plates. After twenty-four hours incubation the colonies were small, about 0·5 to 0·75 mm. in diameter, circular, convex, slightly raised, with an entire edge and a finely granular surface. The centre of the colony was more opaque and a wide zone of β haemolysis was evident round each colony. After forty-eight hours incubation the colonies were a little larger and showed a slightly raised central portion and a flatter periphery. After twenty-four hours incubation in serum broth there was a good growth of organisms, with a granular turbidity and a powdery deposit which disintegrated completely on shaking. There was no surface growth. Seven strains of the organism were examined in detail and were morphologically, culturally and biochemically identical. Morphologically they were typical streptococci, long chained in broth cultures and encapsulated in peritoneal fluid from infected mice.

The organisms fermented the following serum water sugars with the formation of acid and clot, but no gas: glucose, maltose, sucrose, lactose, trehalose and salicin. They did not ferment arabinose, raffinose, inulin, mannitol, sorbitol or dulcitol. Methylene blue in milk was not reduced (Avery, 1929) and a final pH between 5·2 and 5·6 was produced in glucose broth. The organisms did not grow on 10 per cent or 40 per cent bile blood agar and only slight acid and clot was produced in litmus milk. Gelatin was not liquified, nitrates were not reduced, indole and catalase were not formed and sodium hippurate was not hydrolysed (Ayers and Rupp, 1922).

The organisms were found to be sensitive to the following antibiotics, using bacterial sensitivity test tablets: the concentration of the antibiotic per tablet and the diameter of the inhibition zone are given. Penicillin (2·5 IU), 26 to 33 mm; erythromycin (10μg), 22 to 25 mm; terramycin (100 μg), 16 to 22 mm; streptomycin (80μg), 17 to 20 mm; aureomycin (100μg), 14 to 20 mm; neomycin (100μg), 12 to 17 mm. Sulphonamide (1 mg.) gave only a zone of partial inhibition of 19 to 20 mm diameter.

A number of the strains were tested for the ability to lyse human and vervet monkey plasma clots by the method of Tillet and Garner (1933). Each test was carried out in quadruplicate and read at intervals up to twenty-four hours. In a preliminary test one strain only was tested. A human and a healthy vervet monkey served as sources of blood plasma. The organism was fibrinolytic for the human clot in just over one hour and for the vervet clot within eighteen hours. This experiment was repeated using five different strains of the organism from monkeys and one human strain as a control. Two healthy adult humans, one a European (serum 3) and one an African (serum 4), and two vervet monkeys, one an adult (serum 1) and one a youngster (serum 2), served as sources of blood plasma. The results are shown in Table 1. The strains, all of which belonged to Lancefield's group A, were sent for typing to Dr. R. E. O. Williams, Director of the Staphylococcal and Streptococcal Reference Laboratory, Colindale, London, who reported that none of them proved to be typable with his sera.

TABLE I

FIBRINOLYTIC ACTIVITY OF *STR. PYOGENES* FROM VERVET MONKEYS

Strain	*Time in hours required for complete fibrinolysis*			
	Monkey clot		*Human clot*	
	1	*2*	*3*	*4*
107	21	7	5	7
206	nil	12	5	7
222	23	6	6	7
228	nil	12	5	8
386	nil	12	7	8
Human	nil	nil	3	—*

* No clot available.
nil = no lysis.

Pathogenicity

Five strains of the streptococci were each inoculated intraperitoneally into six mice, the dose per mouse being 0·1 ml. of an eighteen hour serum broth culture. All the mice were dead within forty-eight hours, and haemolytic streptococci were isolated from the blood and organs in each case.

One of the strains was tested in the rabbit and guinea pig, in doses of 2 ml. and 1 ml. respectively of an eighteen hour serum broth culture, which proved to be non-pathogenic by intraperitoneal inoculation.

DISCUSSION

In an attempt to establish a human source of infection, throat swabs from all the staff of the Game Department who had had any direct contact with the monkeys were examined bacteriologically. *Str. pyogenes,* however, was not isolated from any of them. The incoming monkeys were therefore examined more carefully and it was seen that a few were sick on arrival, one or two of them showing the typical facial swellings. From one of these, following destruction, *Str. pyogenes* was isolated in pure culture from all organs and the blood. It appears, therefore, that the infection was probably brought from one of the Ssese Islands in the first instance, where it was either acquired from the trappers or their families, or was endemic in the monkey population.

The strains of *Str. pyogenes* isolated from these monkeys are morphologically and biochemically typical human strains, and similar to the strains isolated by Boisvert (1940) from the rhesus monkeys. They do, however, show some points of difference from Boisvert's strains and from normal human strains. Boisvert's strains produced a fibrinolysin which lysed human fibrin but not normal rhesus monkey fibrin, whereas the vervet strains produced a fibrinolysin which lysed both human and normal vervet fibrin, the latter less rapidly. The human control strain of *Str. pyogenes* was slightly more active against human fibrin than the vervet strains, but did not act at all against the vervet fibrin. It appears, therefore, that the vervet strains of *Str. pyogenes*, unlike Boisvert's strains, possess a fibrinolysin capable of lysing the fibrin of their animal host. These vervet strains proved untypable with the typing sera held at the Streptococcal and Staphylococcal Reference Laboratory, London. The differences may possibly indicate that these are "monkey" strains, endemic in the monkey population of one or more of the Ssese Islands and they may later possibly be shown to represent a new type. If these strains are endemic in the wild monkeys it is likely that the lowering of resistance due to transport, overcrowding, altered diet and fighting would contribute to the epidemic nature of the infection.

CONCLUSIONS

An epidemic of septicaemia due to group A haemolytic streptococci (*Str. pyogenes*) in a group of about one hundred vervet monkeys held in captivity in Uganda is described. Approximately thirty deaths occurred, post mortem examinations were performed on fifteen and *Str. pyogenes* was isolated in pure culture from eleven. Treatment with penicillin, and the administration of aureomycin supplement in the food, proved an effective method of control.

The pathological picture seen in the monkeys, the morphological, cultural and biochemical characteristics, the antibiotic sensitivity and fibrinolytic activity of the casual organisms are described. The strains isolated proved untypable with the usual typing sera and they produced a fibrinolysin capable of lysing vervet monkey, as well as human, fibrin. It is suggested that these strains may be endemic in the local wild monkey population and may possibly represent a new type.

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THE INFLUENCE OF SEX ON EQUINE ENCEPHALOMYELITIS IN THE MOUSE AND ON ITS TREATMENT WITH MEPACRINE

By

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INTRODUCTION

In mice the antimalarial drug mepacrine exercises considerable protective effect against a limited number of viral infections (Hurst, Melvin and Peters, 1952; Hurst, Peters and Melvin, 1952), an effect associated with marked suppression of viral growth (Hurst, Melvin, Paget and Snow, 1960). In studying the sequence of events in mice receiving suboptimal doses of the acridine we noticed that with diminishing dosage the protective effect wore off much more quickly in the male than in the female (Hurst, 1957). This observation prompted us to enquire whether the disease itself is inherently more severe in the male and whether or not the phenomena are attributable to hormonal factors. The results of the enquiry are described in what follows.

METHODS

Mice. In all the earlier experiments we used litter-mates chosen at the time of weaning and allowed to grow to sexual maturity. Subsequent statistical analysis, by our colleague Dr. O. L. Davies, of the fate of animals individually identified during the experimental period showed this to be an unnecessary refinement: as in intracerebral assays of pertussis vaccine (Irwin and Standfast, 1955), in which also the response is all or nothing (death or survival), the use of litter-mates led to no appreciable increase in the accuracy of the results. The strains of mice employed have been two, a commercially-available strain and a strain maintained as a closed colony and derived originally from the BSVS strain of the Rockefeller Institute (Webster, 1937). With one exception to be mentioned later, both have furnished essentially similar information.

Infection of mice. At the time of infection, males weighed approximately 25 to 33 g. and females 23 to 31 g.; in any one experiment the weights of the animals of a given sex did not differ by more than 2 g., nor the males from the females by more than the normal sex difference for the age. The degree of maturity indicated by these weights necessitated a large infecting dose of "virulent" virus intramuscularly to ensure a reasonable incidence of encephalitis; that under these circumstances some adult mice escaped encephalitis and death does not imply that they avoided systemic infection with the virus (Hurst *et al.*, 1960). The virus was the strain of Eastern equine encephalomyelitis virus used in previous studies (Hurst, 1950).

Titrations of virus. Mice were bled from the heart to yield pooled defibrinated blood. The vascular system was then washed out with saline until macroscopically the organs and tissues were bloodless; this procedure removed circulating virus at an early stage and circulating antibodies later. Pooled blood or organs were titrated intracerebrally at half-logarithmic intervals to obtain an overall impression of the amount of virus present at various times, or separate organs were examined to bring to light individual variations between animals. Data for the adrenals were obtained in a separate experiment in which groups of 30 mice were not washed out with saline; changes in viral titres here were much too large to be vitiated by the omission. The method of estimating 50 per cent endpoints (not LD_{50}'s) was described by Hurst *et al.* (1960).

Examination of the blood titres (expressed as the negative logarithm to the base 10 of the dilution yielding a 50 per cent endpoint), at 24 and 48 hours after infection in 16 groups each of 6 female mice, showed a significant correlation with the weights of the mice at the time of infection. The regression equations of titre (y) on weight (x) at 24 and 48 hours respectively were:

$$y=5{\cdot}8-(0{\cdot}137 \pm 0{\cdot}067)x \quad (P=0{\cdot}05)$$
$$y=4{\cdot}8-(0{\cdot}141 \pm 0{\cdot}033)x \quad (P=0{\cdot}001)$$

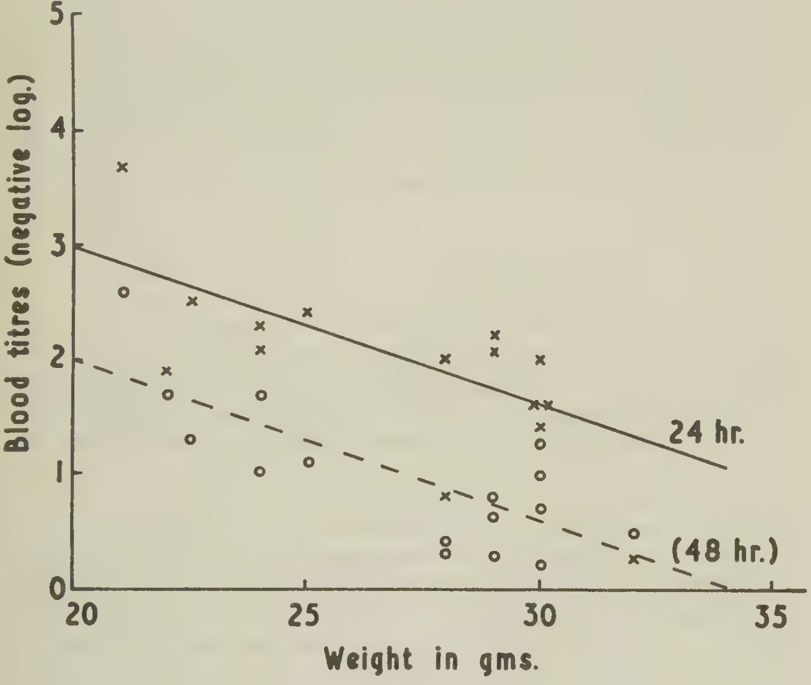

Fig. I

Correlation of viral titres (y) in the blood at 24 and 48 hours after infection with initial weight of the mice in grammes (x)
x 24 hr. o 48 hr.

Correlation coefficients: at 24 hr. r = —0·48 (P = 0·05)
at 48 hr. r = —0·755 (P = 0·001)

Regression equations: at 24 hr. y = 5·8—(0·137 ± 0·067)x
at 48 hr. y = 4·8—(0·141 ± 0·033)x

The observations from which these equations were derived, together with the appropriate regression lines appear in Fig. 1. Scrutiny of the grouped data for blood titres, corrected for variations due to weight and time after infection, revealed a normal Gaussian distribution. In combining the observations from replicate experiments, therefore, the titres of virus expressed as negative logarithms of dilution have been presented as arithmetic means.

Treatments. Mepacrine was given as a single oral dose 24 hours before virus. Hormones, etc. were given subcutaneously daily for 8 days before virus and up to 6 days after. In the earlier experiments the latter were dissolved in arachis oil. We had taken the precaution of testing this vehicle for an adverse influence on mortality from equine encephalomyelitis and in most experiments had obtained no evidence of such an effect. Our colleague Dr. A. L. Walpole had also shown the sample used to be devoid of oestrogenic action. Later, however, in some experiments it appeared that, while having little effect on the untreated infection, arachis oil definitely interfered with the therapeutic properties of mepacrine. Observations on the growth curves of virus in the presence of arachis oil, with and without additional treatment with mepacrine, were wholly consistent with this finding; they will not be recorded in detail in the present communication. The hormonal experiments, therefore, were all repeated using a dispersion of hormone which previously had been ground very finely in a mortar. The dispersing agent (Dispersol OG) has been in use in these laboratories for many years and successive batches have proved non-toxic, even in massive doses, when introduced by a variety of routes.

RESULTS

The Influence of Sex on Mortality

Several years ago we examined the relative susceptibility of male and female mice to encephalitis and death following infection with the virus of equine encephalomyelitis. We then observed no difference between the sexes. At that time, however, because of the more constant invasion of the nervous system in younger animals, we were using mice of around 17 to 18 g. With older animals the case is different.

In 22 consecutive experiments with mice near the lower end of the weight-range used in this investigation (23 to 24 g. female, 25 to 26 g. male), the mortality among male mice exceeded 85 per cent with the large infecting doses of virus used. In 9 experiments male mortality was higher than female, in 9 experiments it equalled the female and in 4 it was below. Over the whole series, 422 of 490 males died and 404 of 490 females. The mean period of survival of the males exceeded that of the females in 5 experiments, equalled it in 4 and was shorter in 13. Over the whole series, the mean period of survival of males was 5·6 days and of females 5·9 days. The latter figures bordered on the significant ($P = 0{\cdot}05$). In 24 consecutive experiments with mice at the higher end of the weight-range (28 to 31 g. female, 30 to 33 g. male), male mortality was greater than female in 21 experiments, equal in 1 and lower in 2. In this series, 254 of 460 males (55 per cent) died and 158 of 460 females (34 per cent). The mean period of survival of the males exceeded that of the females in 8 experiments, equalled it in 1 and was shorter in 15. The mean period of survival of all males was 6·2 days and of females 6·8 days. These differences, and especially those for mortality, are statistically significant. In many experiments the differences in survival-time were brought out very clearly by inspection of the cumulative death-curves.

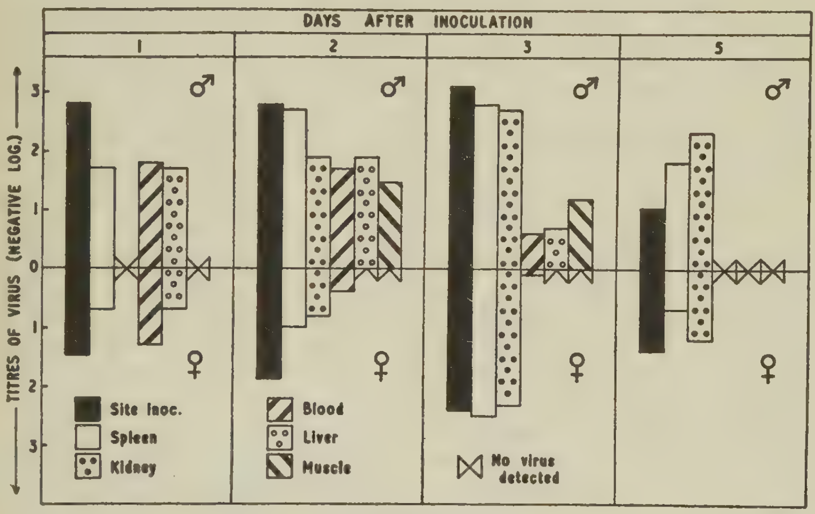

Fig. 2

Mean titres of equine encephalomyelitis virus in the blood and viscera of male and female mice.

The data represent the means of 3 experiments. The mortalities in parallel groups totalling 60 mice of each sex were 38 males and 33 females.

Thus, with increasing maturity of the mouse, a clear difference in the susceptibility of males and females to death from encephalitis emerges. We have previously associated the tendency to encephalitis largely with the degree and duration of viraemia (Hurst, 1950). In the mouse, as in other species, the virus attains far higher titres in the blood and viscera of the younger animal in which mortality from encephalitis is highest. In adults of either sex the systemic infection follows the general trend previously described (Hurst *et al.*, 1960), but at any given age within the range at present under consideration the titres are higher in the male than in the female. Fig. 2 portrays the mean titres obtained in 3 experiments using males of 30 to 33 g. and females of 28 to 31 g. and clearly illustrates this sex-difference. Particularly noteworthy are the earlier disappearance of virus from blood and liver in the female and the much lower frequency with which virus is recovered from muscle away from the site of inoculation. The proportion of individual animals in which virus is recovered from the spleen or kidney at 72, 96 or 120 hours after infection does not differ appreciably between the sexes, but the female titres are lower.

TABLE 1

EFFECT OF TESTOSTERONE, STILBOESTROL AND OESTRADIOL ON EQUINE ENCEPHALOMYELITIS IN A COMMERCIAL STRAIN OF MICE AND ON ITS TREATMENT WITH A SUBOPTIMAL DOSE OF MEPACRINE

Treatment		*No. of mice of each sex*	*Per cent deaths*		*Ratio a/b*	
			(a) Males	*(b) Females*		
No treatment (dispersing agent)		240	75·4	67·0		1·13
Mepacrine	2 mg.	140	60·7	42·9	1·41	1·49
	3 mg.	80	31·3	26·3	1·19	
	4 mg.	100	26·0	14·0	1·86	
Testosterone	0·02 mg.	180	75·0	67·2		1·12
Testosterone + mepacrine	2 mg.	140	60·0	55·5	1·08	1·76
	3 mg.	80	28·8	12·5	2·30	
	4 mg.	100	44·0	23·0	1·91	
Stilboestrol	0·025 mg.	140	65·7	62·1		1·06
Stilboestrol + mepacrine	2 mg.	100	61·0	63·0	0·97	1·07
	3 mg.	40	35·0	30·0	1·17	
	4 mg.	60	30·0	28·3	1·06	
Oestradiol	0·01 mg.	140	70·0	75·4		0·93
Oestradiol + mepacrine	2 mg.	80	90·0	78·8	1·14	1·09
	3 mg.	80	60·0	57·5	1·04	
	4 mg.	100	—	37·0		

The doses given above are per 20 g. body-weight. For regime of dosing see text.

The Effect of Sex upon Treatment with Mepacrine

A single large dose (8 to 10 mg.) of mepacrine almost completely suppresses viral growth and protects nearly all mice against death from encephalitis. The few animals which die do so usually after a long incubation period and represent "escapes" of the virus as the effect of the single dose wears off; nearly all may be protected by a second dose of the drug two days after the first (unpublished observation). At these levels of dosage mepacrine is sufficiently potent to protect animals of both sexes and little or no sex-difference is apparent. It is with suboptimal doses that a differential mortality in favour of the female becomes obvious and is more marked than would be expected from observations on the untreated disease. A differential also exists with treatment of mice of an age at which a sex-difference in the untreated disease is not clearly demonstrable. The result of a typical preliminary experiment has already been reported (Hurst, 1957), and Table 1 includes among other information the results of subsequent more numerous tests. In both sexes a positive dose-response curve to the drug is clearly evident, but whereas the lowest effective dose in males in many experiments is above 2 mg., in females 1 mg. nearly always leads to a distinct lowering of mortality.

The Effects of Testosterone, Stilboestrol and Oestradiol on Mortality

In view of the more successful treatment of the female mouse infected with equine encephalomyelitis, the effects of combining mepacrine with sex hormones and oestrogenic agents was not altogether expected. Table 1 summarises the findings in 8 consecutive experiments in which we studied the action of one or more of the above substances on the untreated disease in both sexes and on the disease treated with a single suboptimal dose of mepacrine. The mice used here came from the commercially available strain; the duration of hormonal treatment has already been indicated. The differential mortality in favour of the female is evident under nearly all circumstances. It is, however, reversed by oestradiol and reduced by stilboestrol, both in the absence of other treatment, and it also appears to be reversed by stilboestrol together with the smallest dose of mepacrine. Oestradiol moderately increases mortality from the untreated disease in the female. For reasons we have been unable to discern, the results have been rather variable from experiment to experiment; often the adverse action is more marked than is suggested in Table 1 and is observed also in the male (see also Table 2). Stilboestrol, on the other hand, tends to reduce mortality in otherwise untreated males and females. Testosterone leaves it unaffected.

TABLE 2

EFFECT OF SEX HORMONES ON EQUINE ENCEPHALITIS IN NORMAL AND GONADECTOMISED MICE OF THE BSVS STRAIN

Treatment	*Deaths in 30 mice*			
	Males		*Females*	
	Normal	*Gonadectomised*	*Normal*	*Gonadectomised*
None	9	6	9	13
Testosterone 0·02 mg.	11	5	8	4
Oestradiol 0·01 mg.	26	28	28	25

As already pointed out, the mean differential in favour of the female in mice treated with mepacrine is greater than would be expected from the difference observed in untreated animals and it is still greater in mice dosed additionally with testosterone. The favourable differential is diminished with stilboestrol or oestradiol and mepacrine. Testosterone may exercise a slightly adverse effect on therapy in both sexes, especially the male, and stilboestrol in the male. In females stilboestrol has a more definitely adverse effect. Oestradiol is markedly disadvantageous in both sexes, to a degree much greater than would be expected from the modestly increased mortality commented on above, but one more in keeping with the results in Table 2. The existence of a dose-response curve with mepacrine in oestradiol-prepared mice suggests, what is in fact the case, that a sufficiently large dose of mepacrine (8 to 10 mg.) can largely overcome the action of the oestrogen.

The difference between oestradiol and stilboestrol in countering the therapeutic action of mepacrine cannot be accounted for on the basis of dosage of the two substances. As an oestrogen, stilboestrol is about one-fifth as active as is oestradiol, so that in our experiments the relative dosage was about one-half. With both substances, however, the dosage was much more than enough to afford a maximal oestrogenic response. Moreover, oestradiol has a malign influence on therapy at much smaller doses (0·001 mg.); incidentally a dose-response curve can be obtained for its capacity to annul the therapeutic properties of mepacrine.

The Effect of Sex Hormones in Normal and Gonadectomised BSVS Mice

This strain of mice differs from the commercially available strain, in that the effect of oestrogen on the untreated disease is relatively greater. Table 2 displays the results of an experiment in which we examined the effects of hormones in normal and in gonadectomised mice. Gonadectomy took place at the age of 4 to 5 weeks, 10 weeks before infection with virus; at the time of infection the gonadectomised females were about 10 per cent heavier than their controls, but the gonadectomised males were of the same weight as their controls.

The identity of mortality in intact untreated males and females in Table 2 is not characteristic of this strain of mice. In other experiments involving 180 adult mice of each sex, male mortality amounted to 47 per cent and female to 19 per cent. Slight apparent effects of gonadectomy in reducing mortality in the male and enhancing it in the female were doubtfully significant; the trend was not evident in a second experiment, nor was the apparently beneficial effect of testosterone in the gonadectomised female. The disadvantageous action of oestradiol on the otherwise untreated disease in both normal and gonadectomised animals is in the same direction as, but wholly different in degree from, that observed in the commercial strain of mice. It was confirmed in a second experiment and was seen equally in gonadectomised animals of both sexes. In BSVS mice we also obtained suggestions, not statistically significant, that female mortality of the untreated disease might be lower, and treatment with mepacrine more successful, during anoestrus rather than in the opening phases of the oestrus cycle.

Other Substances Examined

A third oestrogen, tri-*p*-anisylbromoethylene, induces continuous oestrus of considerable duration when given to mice in a single dose of 0·1 mg. subcutaneously. In two experiments in intact female mice it produced no conclusive effect either on infection with equine encephalomyelitis or on its treatment with mepacrine.

Gestyl (gonadotrophin from pregnant mares' serum) and Pregnyl (human chorionic gonadotrophin) act as follicle-stimulating agents. In two experiments in the intact female infected with equine encephalomyelitis, daily administration of 2 units of each resulted in a much heavier mortality than in the controls. The protective action of 2 and 3 mg. mepacrine was completely abolished. As might have been predicted, no such action was observed in gonadectomised females or in intact males. Progesterone (1 mg. daily) did not influence the disease or its treatment with mepacrine in either males or females.

2-4-dinitro-4′-methyldiphenylamine slightly inhibits the vaginal response of castrated animals to oestrogens. In intact animals it inhibits completely the cycle of vaginal change. It can, however, be regarded as only partially "anti-oestrogenic". In three experiments in which it was given at a dose of 1 mg. twice daily intraperitoneally, it had little effect on mortality from equine encephalomyelitis in the male but increased it by 25 per cent in the female. It very largely abolished the action of 2 and 3 mg. mepacrine in both sexes.

The Growth of Virus under the Influence of Hormones, etc.

Oestradiol. We administered the hormone to unmated adult female mice of the commercial strain; in different experiments they ranged from 25 to 30 g. in weight at the time administration of the oestrogen began, 8 days before infection. A large intramuscular dose of virus (10^{-2}) to these animals resulted in death from encephalitis in rather more than 50 per cent; the remainder suffered an inapparent systemic infection. Treatment with oestrogen caused the mice to gain weight more rapidly than the controls, so that at the time of infection they were about 10 per cent heavier; this fact was taken into account in calculating the dose of mepacrine. Various organs (e.g. kidneys) were also about 10 per cent heavier than those of the controls; the spleens and the adrenals were 50 per cent heavier. In the treated animals, the organs were rendered bloodless with greater difficulty than in controls, and vascular irrigation needed to be more prolonged.

Fig. 3 presents the more important titres of virus in the blood and tissues of female mice under the influence of oestradiol and treated or not with mepacrine, compared with those of corresponding controls receiving no oestradiol. The figures are the means of 5 separate experiments all of which showed the same trends; the total data warrant the following conclusions.

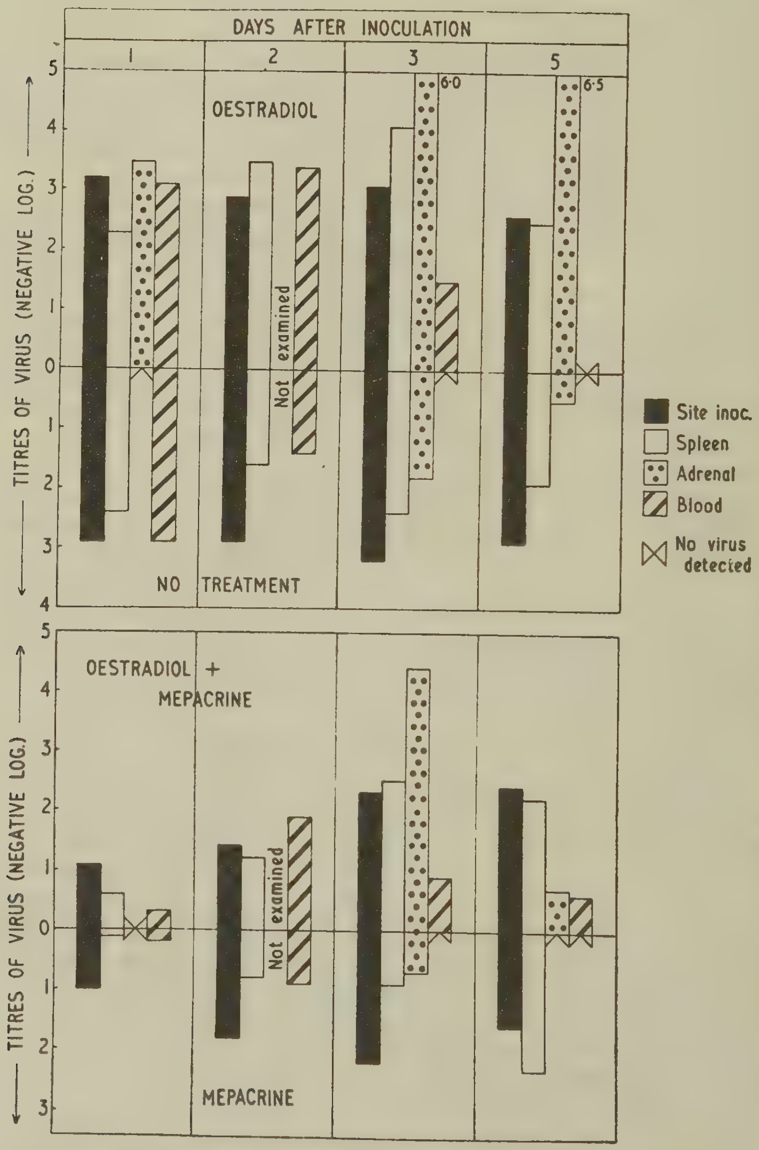

Fig. 3

Mean titres of equine encephalomyelitis virus in the blood and organs of female mice treated or untreated with oestradiol or mepacrine.

The data represent the means of 5 experiments. Oestradiol was given as in Table I. The single oral dose of mepacrine was of 200 mg./kg. The mortalities in parallel groups totalling 80 mice were as follows: no treatment 45, mepacrine 10, oestradiol 60, oestradiol plus mepacrine 29.

Oestradiol exerts no appreciable effect on the titres of virus at the site of inoculation in animals not receiving mepacrine. It also does not influence the partial inhibitory effect of the suboptimal dose of mepacrine until possibly late on, when no doubt the effect of the acridine is waning. As reported elsewhere (Hurst *et al.*, 1960) voluntary muscle away from the site of inoculation furnishes very variable results; the number of isolations of virus was rather greater in the oestradiol-treated mice, but not in those receiving mepacrine as well. Isolations of virus were also more frequent from cardiac muscle of mice given oestradiol. Quite consistently at 24 hours, the titre of virus in the blood was virtually identical with oestradiol and without. Thereafter the rapid fall in the untreated animal was replaced by a sustained and even increasing (4 of 5 experiments) viraemia in the oestradiol-treated group, so that the intensity and total duration of the viraemia greatly exceeded that in the controls. Mepacrine as usual considerably diminished viraemia and oestradiol cancelled this effect in no slight degree. As always, the curves for virus in the liver closely followed those for the blood, but at a rather lower level.

At 24 hours splenic virus was the same in the oestradiol-treated group as in controls; thereafter virus consistently rose to much higher levels with the oestrogen. Not only, therefore, were the spleens of these animals heavier—they also contained more virus per unit-weight. The rising titres in the later stages in mice dosed with mepacrine were largely accounted for by animals in which the infection "escaped" after temporary control by the drug. Here again, the inhibitory effect of mepacrine was reduced by prior medication with oestradiol. The relative numbers of infective and non-infective spleens at 72 and 96 hours were similar in male and female mice treated with oestradiol and were greatly in excess of those in mice not receiving the hormone. At 120 hours this preponderance had been reversed. The titre of virus in the kidney was not clearly altered by oestradiol.

In mice of the size used in these experiments, we have not noticed the considerable proliferation of virus reported by Traub (1958) as occurring in the bone-marrow. Under the influence of oestradiol, however, growth here was much stimulated. Whereas controls at 24, 48 and 72 hours gave titres below 10^{-1}, the corresponding figures in oestradiol-treated mice were $10^{-2\cdot 9}$, $10^{-2\cdot 4}$ and $10^{-2\cdot 1}$. In the ovaries virus persisted at a higher level in the oestradiol-treated mice; the relevant titres were: controls $10^{-2\cdot 1}$, 10^{-2}, 10^{-1}; oestradiol-treated $10^{-2\cdot 5}$, 10^{-3}, $10^{-3\cdot 2}$, at 24, 48 and 72 hours respectively.

The most marked effect of oestradiol was on virus in the adrenals. Here, viral growth was very greatly stimulated, whether mepacrine was combined with the hormone or not; without the acridine, maximum titres exceeded 10^{-6}. Virus still persisted ($10^{-1\cdot 5}$) in the adrenals of oestradiol-treated mice at the latest time at which observation was made, viz. 168 hours. Definite histological changes resulted from exhibition of the hormone. The cortical cells of the innermost zona fasciculata were greatly swollen, pale and excessively vacuolated, with eccentric nuclei. The sinuses were dilated and engorged with blood, and in extreme cases they stretched and distorted adjacent glandular cells; their endothelium was swollen and proliferating and occasionally polymorphs lay in the surrounding tissues. In some animals similar lesions spread inwards into the X-zone, which was still present, and in this zone hyper-eosinophilic elements with pyknotic or fragmented nuclei occurred far more frequently than in control organs. In a minority of mice infected additionally with virus, massive acute necrosis and infiltration with polymorphonuclears, many karyorrhectic, represented still more serious damage to this gland.

Stilboestrol. A single titration with this substance in female mice revealed certain differences from the foregoing. This oestrogen exerted no clear influence on the weight of the animals, or on that of their kidneys or adrenals. Contrary to reports in the literature (Ellinger, 1954), the spleens were about 16 per cent lighter than those of the controls. Histologically, however, the adrenals were more severely damaged than with oestradiol. With stilboestrol alone, the width of the area of affected zona fasciculata and X-zone was greater than with oestradiol, and the frequency and extent of necrosis in additionally infected animals exceeded those in the preceding groups. At the site of inoculation, titres of virus were considerably lower, on an average by 1·0 log. unit, than in controls. The partial suppressive action of mepacrine, however, appeared to have been neutralised to a significant degree. No difference was detected in voluntary muscle away from the site of inoculation.

In the organs, some delay occurred in the rise of viral titres. In the blood, liver and spleen, titres were well below those in the controls at 24 hours, though in all at 48 hours and later there followed the exaggerated rise and persistence of virus which characterises the action of oestradiol. Titres of virus in the kidney were, if anything, lower in the stilboestrol-treated group. Except in the kidney the effect of mepacrine on viral titres was moderately diminished by stilboestrol. In spite of the more severe histological changes in the adrenals, the growth of virus, though increased, was not affected quite so drastically as by oestradiol; virus attained maximum titres of $10^{-4 \cdot 7}$ for stilboestrol and $10^{-3 \cdot 3}$ for stilboestrol plus mepacrine, and in the former group a titre of adrenal virus of $10^{-3 \cdot 9}$ persisted at 168 hours.

Testosterone. We performed two titrations in adult male and two in unmated adult female mice at the upper limit of the weight-range. The steroid appeared to retard increase of weight in the males but not in the females. In both sexes the spleens were about 12 per cent heavier than in the controls, the kidneys slightly heavier (10 and 5 per cent respectively) and the adrenals unchanged in weight. As usual, titres generally were higher in the male animals. In neither sex did the hormone induce any clear change in the viral content of blood, liver, site of inoculation or voluntary muscle. In both sexes it appeared to depress viral titres in the spleen at 48 hours and later; at 24 hours the opposite appeared to be true though the differences were not very great. In both sexes there was clear retardation of viral increase in the kidneys during the first 3 days, though later the titres rose to the level of those in the control groups. Although in the female mice administration of the steroid caused the X-zone to disappear from the adrenals, this disturbance apparently did not promote greater multiplication of virus in the organ and titres were similar to those in control animals. The effects on the growth of virus in mepacrine-treated animals were not sufficiently definite to be significant.

Absence of a Sex Effect in the Guinea-pig

In the guinea-pig we could detect no effect of sex on equine encephalomyelitis. In three experiments with totals of 60 animals of each sex, 36 animals died in each group. In two of these experiments pooled blood was examined at daily intervals without detecting any difference in viral titre between males and females. The animals weighed approximately 500 g. each.

DISCUSSION

The foregoing observations demonstrate a clear sex-difference in the prophylactic action of mepacrine against equine encephalomyelitis in the mouse. A sex-difference is much less obvious in the untreated infection. Although differing toxicity of certain drugs for male and female animals is well recognised, we were not able to detect an appreciably higher toxicity of mepacrine for either sex (Hurst, 1958). By implication, therefore, since differential toxicity often represents different rates of metabolic transformation of a drug in the sexes, it is unlikely that a marked sex-difference exists in the metabolism of mepacrine. The best-studied examples of differential toxicity for the sexes have usually been shown to be under hormonal control, in that they are influenced by the application or withdrawal of one or other sex-hormone, or of both. In this respect the present observations are difficult to explain. In males and females, intact or gonadectomised, administration of the hormone of the naturally more resistant, female sex has a damaging effect, even when given in doses much smaller than those employed in the majority of our experiments. Testosterone, on the other hand, has but little effect on therapy or, if anything, may be slightly deleterious; it induces no obviously increased growth of virus. The growth curves of virus under the influence of oestradiol provide a ready explanation for the greater severity of the disease in its presence; what at the moment is obscure is the greater success of therapy and the rather higher natural resistance of the female not under additional hormonal treatment.

The fact that oestradiol has little effect on the growth of virus at the site of intramuscular injection shows that virus here is not the origin of the increased and more persistent viraemia in mice receiving the hormone. In the liver also, as in the normal animal (Hurst *et al.,* 1960), the titres of virus merely mirror those in the blood and wax and wane with them. Virus levels in the kidney do not appear to be materially altered by oestradiol. In the enlarged spleen, on the other hand, as in the bone-marrow, viral titres are considerably higher (per unit weight of the organ), and in the adrenals higher by as much as four log. units. Virus persists at a high titre longer in the ovaries. Oestrus per se does not necessarily lead to increased mortality, for continuous oestrus evoked by tri-*p*-anisylbromoethylene does not conclusively influence the disease or its treatment with mepacrine.

Oestrogens have previously been reported to confer increased resistance to vaccinia virus injected intradermally in rabbits (Sprunt, McDearman and Raper, 1938; Sprunt and McDearman, 1940) and poliomyelitis virus instilled intranasally in mice or monkeys (Foley and Aycock, 1945; Curley and Aycock, 1946) or fed to mice (Anderson and Bolin, 1946). The phenomena here concerned are almost certainly local rather than systemic; indeed with poliomyelitis the enhanced resistance does not obtain against virus injected intracerebrally or intraperitoneally. Influenza virus is said to reach higher titres in mice given testosterone and lower titres in castrated animals (Kalter, Stuart and Tepperman, 1950; Kalter, Smolin, McElhaney and Tepperman, 1951), apparently thus moving in the same direction as protein anabolism. In hamsters infected intracerebrally with poliomyelitis, heavy dosage with testosterone increases resistance, while recent castration increases susceptibility to virus introduced intraperitoneally (Teodoru and Shwartzman, 1954; Teodoru, 1956). At the moment no common pattern of response to the sex hormones appears to emerge from the data available.

CONCLUSIONS

In the mouse, but not in the guinea-pig, infected with equine encephalomyelitis, adult males are more susceptible to encephalitis and death than are adult females. At any given age within the range considered in this report, the greater susceptibility is associated with higher viral titres in the blood and organs.

Treatment with doses of mepacrine which only partially suppress the growth of virus accentuates the sex-difference and reveals it at an age when it is not clearly demonstrable in the untreated mouse. The difference is enhanced by additional treatment with testosterone.

The differential mortality in favour of the female is reversed by oestradiol and diminished by stilboestrol in a commercially available strain of mice. In this strain, stilboestrol tends to reduce mortality from encephalitis and oestradiol to increase it in females and sometimes in males. In the BSVS strain oestradiol markedly increases mortality in normal and in gonadectomised males and females. Testosterone has but little effect on mortality of the untreated disease.

Testosterone may exert a slightly adverse effect on therapy with mepacrine in both sexes, and stilboestrol in the male. The latter is more definitely damaging in the female. Oestradiol is markedly disadvantageous to therapy in intact and gonadectomised males and females. Gestyl and Pregnyl have a similar action in intact females, but not in gonadectomised females or in males.

A long-acting oestrogen, tri-*p*-anisylbromoethylene, produced no conclusive effect on the disease or on its treatment: progesterone also was inactive. A partial "anti-oestrogen", 2-4-dinitro-4′-methyldiphenylamine, increased mortality in the female and abolished the action of mepacrine in both sexes.

Growth curves of virus in the blood and viscera of animals treated or not with hormones and/or mepacrine showed that whereas testosterone has but little effect, oestradiol and stilboestrol markedly stimulate the growth of virus in certain, but not all, situations and partly overcome the suppressive action of mepacrine on viral growth. Details of their behaviour are described and discussed.

Although injections of arachis oil usually do not clearly affect mortality from equine encephalomyelitis, they go far towards counteracting the therapeutic action of mepacrine. Hormones should therefore be administered in a dispersing agent rather than in oily solution.

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THE INFLUENCE OF CORTISONE, ACTH, THYROXINE AND THIOURACIL ON EQUINE ENCEPHALOMYELITIS IN THE MOUSE AND ON ITS TREATMENT WITH MEPACRINE

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INTRODUCTION

An earlier paper (Hurst, Melvin and Thorp, 1960b) described the influence of sex on infections with equine encephalomyelitis virus in the mouse, and on their prophylaxis with a suboptimal dose of mepacrine. Concomitant administration of certain oestrogens markedly stimulated the growth of virus in some, but not all, situations, and was definitely disadvantageous to therapy. Gestyl and Pregnyl had a similar action in the presence of the ovaries. Testosterone exerted but little effect. It now seems of interest to consider the influence of other hormonal factors on the infection and its treatment, and the present report covers the behaviour of cortisone, ACTH, thyroxine and thiouracil.

EXPERIMENTAL

Methods were those described in the paper referred to above except for the duration of some of the treatments. These were as follows: Oestradiol 0·01 mg. or thyroxine 0·06 mg., subcutaneously, daily for 8 days before and up to 6 days after virus. Thiouracil 0·5 mg., subcutaneously, daily for 10 days before and up to 6 days after virus. Cortisone 0·5 mg., subcutaneously, daily for 2 days before and 4 days after virus. ACTH 0·5 unit, subcutaneously, twice daily for 3 days before and up to 6 days after virus; the material was prepared according to the method of Bruce, Parkes and Perry (1952). Mepacrine 3 or 4 mg. once, orally, 24 hours before virus.

RESULTS

The Effect of Hormonal Influences on Mortality and Time of Survival

Table 1 summarises the results obtained in 10 experiments on fully adult mice using cortisone, thyroxine or thiouracil, together with oestradiol for purposes of comparison. In the control groups the male: female mortality ratio was more disadvantageous to the males than that presented in Table 1 of the preceding paper, though it agreed well with the figures cited in the text of that paper for deaths among mice of the higher weights used. As usual the sex-difference was enhanced by the suboptimal dose of mepacrine. Once again it was abolished by oestradiol which, in the present experiments, considerably increased mortality in both sexes. In otherwise untreated animals, cortisone and thyroxine increased mortality to a still greater degree and abolished the differential in favour of the female; both acted to counter the prophylactic effect of mepacrine. With the dosage of cortisone given in Table 1, the beneficial effect of mepacrine could be only partially restored by increasing the dose of the acridine to 8 mg. With one-tenth the dose of cortisone, increased mortality was still evident in untreated animals; it could be eliminated in females, but not wholly in males, by a dose of 3 to 4 mg. mepacrine. Thiouracil tended to diminish mortality from the untreated disease in both sexes, leaving the male: female ratio and the prophylactic effect of mepacrine but little altered.

TABLE I

EFFECT OF VARIOUS HORMONAL INFLUENCES ON EQUINE ENCEPHALOMYELITIS AND ON ITS TREAMENT WITH A SUBOPTIMAL DOSE OF MEPACRINE

Treatment		*No. of mice of each sex*	*Per cent deaths*		*Ratio a/b*
			(a) Males	*(b) Females*	
No treatment (saline)		200	64·0	40·5	1·58
Mepacrine	4 mg.	70	15·7	7·1	2·21
Oestradiol	0·01 mg.	125	73·6	76·8	0·96
Cortisone	0·5 mg.	175	96·6	98·3	0·98
Cortisone + Mepacrine		70	58·6	47·1	1·24
Thyroxine	0·06 mg.	180	92·2	91·7	1·01
Thyroxine + Mepacrine		80	—	40·0	—
Thiouracil	0·5 mg.	125	44·8	32·0	1·40
Thiouracil + Mepacrine		80	—	7·5	—

The doses given above are per 20 g. body-weight. For regime of dosing see text.

The figures in Table 1 do not bring out one important difference in the behaviour of the steroid hormones on the one hand, and of thyroxine on the other. This difference appears in the cumulative death curves in Fig. 1. While with oestrogen and cortisone the curves are superimposable, and the mean periods of survival of animals succumbing to the infection are only moderately shortened as compared with those of the controls, thyroxine, with slightly less effect on total mortality than cortisone (Table 1), is responsible for major shortening of the time of survival. A large proportion of thyroxine-treated mice die as early as the 3rd day, and 90 per cent of fatalities occur by the 4th day; after the 5th day the curve is virtually level. Conversely, in addition to lowering mortality, thiouracil slightly increases the mean period of survival.

The Growth of Virus under the Influence of Hormones

Cortisone. We performed three titrations using adult male mice (31 to 33 g.), adult unmated females (29 to 31 g.) and young unmated females (20 to 21 g.). Allowing for the normal differences between males and females, and between young and old animals (Hurst, Melvin and Thorp, 1960b), the curves of viral growth showed the same general trends; Fig. 2 displays some representative mean titres, and the full observations are described below. The mortalities in parallel groups are indicated beneath the figure. The number of individual mice carrying virus in certain organs during the later stages of infection was determined in a separate experiment, with the results appearing in Table 2; owing to a shortage of mice at the critical times they are not quite complete, but the general conclusions to be drawn are evident.

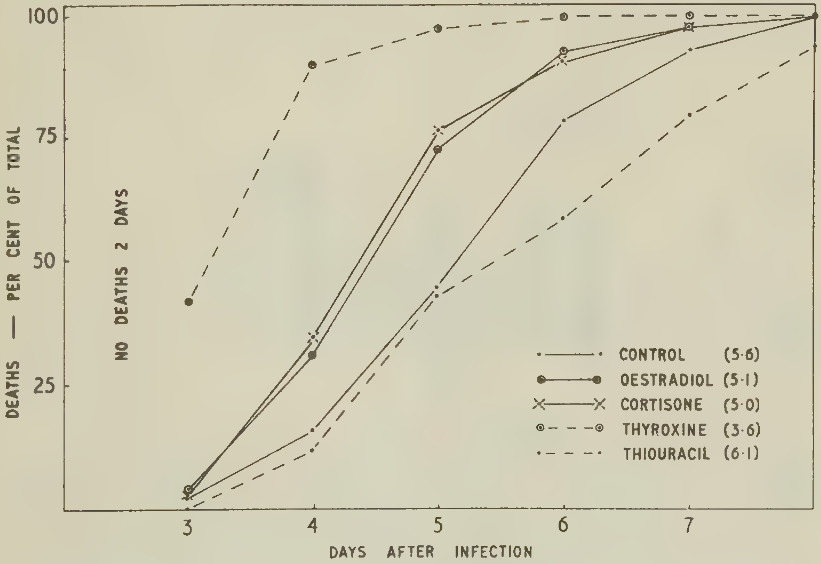

Fig. 1.

Cumulative death-curves in groups totalling 125 male and 125 female mice under various hormonal influences and infected with the virus of equine encephalomyelitis.
The total mortality on successive days is expressed as a percentage of the final mortality in each group. The figures in parentheses are the mean periods of survival (in days) of those animals which died.

Initially, cortisone slightly retarded growth of virus at the site of inoculation but, after dosing with the hormone ceased, virus continued at a higher level here than in the controls. It did not favour the growth of virus in voluntary muscle away from the site of

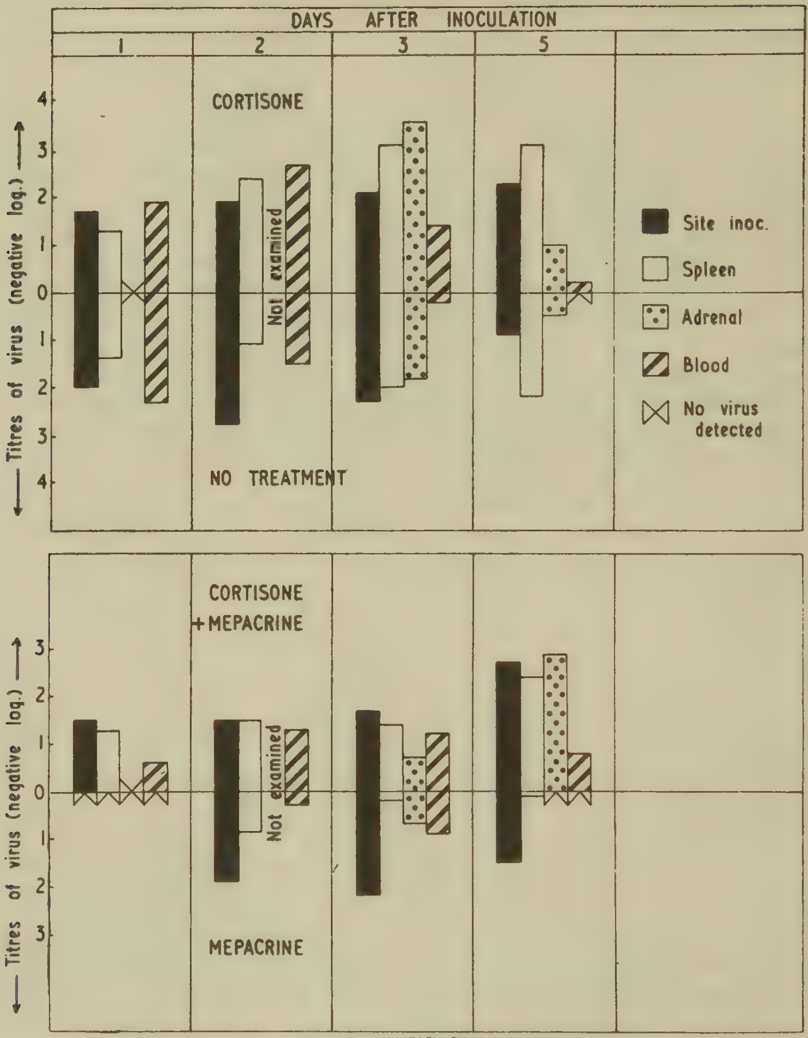

FIG. 2

Mean titres of equine encephalomyelitis virus in the blood and organs of mice treated or untreated with cortisone or mepacrine.

The data represent the means of three experiments in adult male, adult female, and young female mice respectively. Cortisone and mepacrine were given as in Table 1. The mortalities in parallel groups totalling 80 mice were as follows: no treatment 51, mepacrine 7, cortisone 71, cortisone and mepacrine 42.

inoculation; indeed, any difference was in the reverse direction. However, much higher titres obtained in cardiac muscle and, as Table 2 shows, virus was detected much more frequently here than in controls or in oestradiol-treated mice. The hearts of animals dosed with mepacrine never yielded virus, whereas those of animals receiving cortisone in addition were often positive, and the average titres in the cardiac muscle were nearly as high as without the acridine.

In the blood, and in the liver which closely parallels the blood (Hurst, Melvin, Paget and Snow, 1960a), results during the first 24 hours were irregular. In two experiments titres in cortisone-treated animals were lower, in the adult male group higher, than in controls; the mean figure for the three experiments gave a slightly lower titre for cortisone. Thereafter, however, in both intensity and duration viraemia exceeded that in controls. The steroid also largely cancelled the suppressive effect of mepacrine.

TABLE 2

PRESENCE OF VIRUS IN INDIVIDUAL ORGANS IN MICE TREATED OR UNTREATED WITH HORMONES, ETC.

Treatment	*Heart*			*Spleen*			*Kidney*		
	72 hr.	*96 hr.*	*120 hr.*	*72 hr.*	*96 hr.*	*120 hr.*	*72 hr.*	*96 hr.*	*120 hr.*
Control (saline)	22·5	12·5	0·0	65·0	30·0	40·0	42·5	25·0	27·5
Oestradiol 0·01 mg.	50·0	15·0	—	—	67·5	20·0	—	32·5	7·5
Cortisone 0·5 mg.	82·5	80·0	80·0	—	85·0	80·0	—	82·5	82·5
Thyroxine 0·06 mg.	65·0	32·5	—	77·5	45·0	17·5	60·0	30·0	27·5
Thiouracil 0·5 mg.	17·5	10·0	0·0	—	35·0	20·0	—	25·0	15·0

Groups of 20 males and 20 females, fully adult and of similar age-groups, received various treatments as detailed in the text. Individual organs were examined for the presence of virus at the times stated. The figures above are the percentage positive in each group.

Splenic virus was not demonstrably altered by cortisone during the first 24 hours. Later, titres with cortisone were definitely higher, and the spleen was infective in a greater proportion of individual animals than among controls or among mice given oestradiol. Moreover, the more frequent disappearance of virus from the spleens of oestradiol-treated animals at 120 hours (Hurst *et al*, 1960b) was not duplicated with cortisone. The inhibitory effect of mepacrine on splenic virus was considerably reduced. In all instances, titres were of course in terms of unit-weight of the organs which, in the cortisone-treated mice, were only 15 to 25 per cent as heavy as controls; the spleens of the mepacrine plus cortisone group were 35 to 40 per cent of those of animals receiving only mepacrine. The last did not differ from untreated controls. Lymph nodes were not titrated but, on the basis of the period of incubation and number of fatalities in passage animals given a 1:100 suspension, virus was not present in greater amount or more frequently after administration of cortisone than without. Cortisone increased the amount of virus present in the bone-marrow at 24, 48 and 72 hours; the titres were about half a log. unit below those with oestradiol in the earlier paper. Titres in the lung were higher and virus persisted longer with cortisone, and the complete suppression of pulmonary titres by mepacrine was largely abolished.

In equine encephalomyelitis, virus in the kidney normally appears only in the later phases of the infection (Hurst *et al.,* 1960b). It rose more slowly in cortisone-treated mice, but by 120 hours had reached a higher titre and was present in a greater proportion of individuals. The kidneys were 13 to 24 per cent heavier than those of the controls. The effect of mepacrine on viral titres was partly neutralised. It will be recalled that oestradiol was without demonstrable effect on renal virus.

In one experiment cortisone apparently increased testicular virus at 72 hours—none was detected at other times. In the ovaries at 24, 48 and 72 hours an end point was not reached at 10^{-4}, giving a level of virus at least 100 times that in controls.

Virus in the adrenals was studied in the same separate experiment as oestradiol, stilboestrol, etc. (Hurst *et al.,* 1960b), so that the figures are directly comparable with those. Although virus grows much better here in the presence of cortisone (Table 3), it does not reach anything like the levels attained with oestradiol. In contrast to the latter and to ACTH, no virus was detected at 24 hours; in both the cortisone group and that receiving mepacrine in addition, however, virus persisted in the adrenals at 168 hours as it did with oestrogens and with ACTH. Histologically, cortisone did not produce the severe morphological changes seen with oestradiol, nor was adrenal necrosis present in mice receiving virus in addition.

While, therefore, cortisone shows many features in common with oestradiol, it differs in having less effect on virus in the adrenals, a greater effect on virus in the kidney and ovaries, and, on the basis of persistence of virus in individual animals, a greater effect in the spleen and heart. As all these effects are not in the same direction they probably cannot be explained by different schedules of dosing of the two hormones. Since death in equine encephalomyelitis results from invasion of the brain by the virus, and this event apparently depends on the degree and duration of the viraemia (Hurst, 1950), and since the viraemia in the present experiments was not more extreme than in those previously recorded with oestradiol (Hurst, *et. al.,* 1960b) it is not at all certain that the above-mentioned differences in the action of the two hormones are directly concerned in the much heavier mortality obtaining in animals receiving cortisone.

ACTH. We carried out only a single titraticn in young female mice. The results were compared both with the mean experiences with cortisone as related above, and with the individual results in the cortisone experiment in young females. The mortalities in parallel groups of 20 mice held under observation were: controls 17, ACTH 18, mepacrine 4, ACTH+mepacrine 8. As is obvious, in mice of the age used the controls suffered a heavy mortality—this was the case also in the comparable experiment with cortisone—and deaths among those receiving ACTH were of the same order. The combination of ACTH with mepacrine was responsible for a modest increase in mortality over that ensuing with mepacrine alone, whereas in the comparable experiment with cortisone, the steriod had increased the deaths from 2 to 15, and in other cortisone experiments neutralisation of the beneficial effects of mepacrine was equally marked. At the site of inoculation in the ACTH-treated group, virus was apparently increased at 48 hours but not at other times. The suppressive effect of mepacrine on viral growth was scarcely affected. Viral titres in voluntary muscle generally were not increased and may have been decreased by the hormone. Cardiac muscle was not examined.

TABLE 3

EFFECT OF VARIOUS HORMONES ON THE TITRE OF EQUINE ENCEPHALOMYELITIS VIRUS IN THE ADRENALS TREATED OR UNTREATED WITH MEPACRINE

Treatment	*Titres of Virus (negative log.)*			
	24 hr.	*72 hr.*	*120 hr.*	*168 hr.*
Controls	N.V.	1·8	0·5	N.V.
Mepacrine 4 mg.	N.V.	0·7	N.V.	N.V.
Oestradiol 0·01 mg.	3·5	6·0	6·5	1·3
Oestradiol + Mepacrine	N.V.	4·4	0·7	N.V.
Cortisone 0·5 mg. ..	N.V.	3·6	4·1	0·7
Cortisone + Mepacrine ..	N.V.	0·7	2·9	1.1
ACTH 0·5 unit	2·8	4·1	3·8	3·2
ACTH + Mepacrine	2·5	3·8	4·4	3·1

The doses given above are per 20 g. body-weight. For regime of dosing see text.
N.V.=no virus detected.

As against the average result with cortisone, and the result with this substance in young female mice, virus in the blood (and liver) was clearly raised by ACTH during the first 24 hours of infection; it will be remembered that in one experiment with cortisone a similar early rise occurred in the adult male group. Thereafter, as with cortisone, virus circulated longer and at a higher level than in controls. The relevant titres for control and ACTH mice were: 24 hours 1·9 and 2·6, 48 hours 1·7 and 2·2, 72 hours 0·4 and 1·6, and 96 hours nil and 0·2. The suppressive effect of mepacrine was not greatly changed. During the first 24 hours, splenic titres also rose well above those in control animals, and they remained well above at all subsequent times; the effect of mepacrine persisted in the presence of ACTH.

The most pronounced differential effect of ACTH vis-à-vis the other hormones studied concerned viral multiplication in the adrenals (Table 3), as determined in the separate experiment to which reference has already been made. At 24 hours no virus was detected here in control or cortisone-treated animals, but, as also with oestradiol, abundant virus was present at this time in mice receiving ACTH. With both oestradiol and ACTH the adrenals weighed about 50 per cent more than the controls, whereas with cortisone the adrenals were 10 per cent lighter. At 72 and 120 hours adrenal titres were about the same as with cortisone, and far lower than with oestradiol, but a high titre of virus continued at 168 hours when the titres with other hormones had fallen considerably. Whereas in blood, liver, spleen and site of inoculation, ACTH had little effect on the inhibition of viral increase by mepacrine, in the adrenals of ACTH-treated mice the acridine had no appreciable effect. With oestradiol and cortisone, on the other hand, mepacrine was still able partially to counteract the stimulative action of the hormone.

To summarise, ACTH differs from both oestradiol and cortisone in its action on mepacrine-treated infections with the virus of equine encephalomyelitis. Although clearly stimulating the growth of virus in several situations, it does not easily overcome the inhibitory effect of mepacrine on viral growth except in the adrenals; here it completely abolishes the effect of the acridine.

Thyroxine and thiouracil. The results depicted in Fig. 3 derive from two titrations for each substance in fully adult, unmated female mice, each with its own controls and with additional titrations in mepacrine-treated groups. In the thyroxine titrations all the figures were higher than in the controls except for two readings at the site of inoculation and for the spleens at 72 and 96 hours, when many of the thyroxine-treated mice were already dead. In the thiouracil titrations all figures were the same as or lower than those in their controls except for a solitary reading at the site of inoculation. In Fig. 3, therefore, the control data have been omitted and the thyroxine results have been contrasted directly with the thiouracil. Deaths among parallel groups held under observation are indicated below the figure. The numbers of mice carrying virus in individual organs have already been included in Table 2. The data for groups treated additionally with mepacrine have also been omitted from Fig. 3. Those for thyroxine and mepacrine closely resembled those for cortisone in Fig. 2 in that the hormone partially removed the inhibitory effect of the acridine on viral multiplication. Thiouracil made no appreciable difference to the action of mepacrine. Thyroxine caused the mice to gain total weight much more slowly than the controls, but the weights of the heart and kidneys were increased by about a third, and of the spleens by about one-sixth.

At the site of inoculation thyroxine did not convincingly influence viral growth while thiouracil, initially at any rate, gave rather lower titres as compared with its controls. In voluntary muscle generally, however, virus was isolated more constantly and in much higher titres with thyroxine, and cardiac muscle was even more affected.

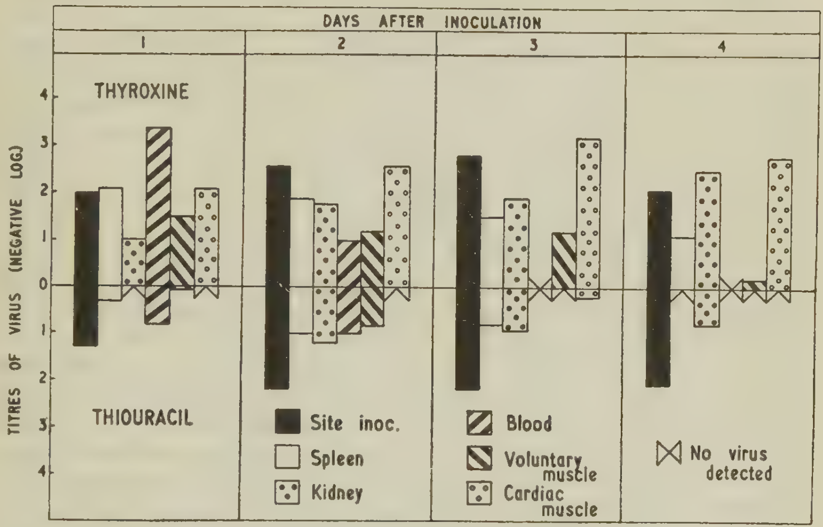

Fig. 3

Mean titres of equine encephalomyelitis virus in the blood and tissues of mice treated with thyroxine or thiouracil.

The data represent the means of two experiments with each substance in adult female mice. The mortalities in parallel groups totalling 40 mice for each substance were:

No treatment	26	No treatment	18
Mepacrine	6	Mepacrine	3
Thyroxine	33	Thiouracil	16
Thyroxine + Mepacrine	15	Thiouracil + Mepacrine	3

Viraemia was much more pronounced with thyroxine, but whereas with oestradiol and cortisone the increase occurred after the normal peak of viraemia, with thyroxine it occurred as a gross exaggeration of the normal and the duration of viraemia was the same as in controls. The period after inoculation during which virus is absent from the bloodstream is so brief (Hurst, *et. al.*, 1960a) as to exclude an appreciable increase in the duration of the viraemia at an earlier stage. With thiouracil, on the other hand, virus in the blood was lower than in controls; moreover the peak of viral circulation was delayed and was attained only on the second day.

Viral titres in the spleens of thyroxine-treated mice were much higher than in controls at 24 and 48 hours; thereafter, in the rapidly diminishing number of survivors, they were lower. At a late stage the earlier, very frequent isolation of virus from individual spleens gave place to the opposite state of affairs (Table 2). At 24, 48 and 72 hours the titres of virus in the spleens of animals receiving thiouracil were lower than those in the corresponding control mice. Growth of virus in the bone-marrow was greatly augmented by thyroxine. The titres at 24, 48 and 72 hours were $10^{-4\cdot1}$, $10^{-2\cdot1}$ and 10^{-1} respectively; control figures were below 10^{-1} on all occasions.

At all times renal titres were raised by thyroxine; virus was present at 24 hours, which is not normally the case, and continued to rise throughout the period of observation. In controls and in animals given thiouracil virus did not appear in the kidney until the second day and, in the latter, titres were consistently lower than in controls. In the earlier stages virus was also isolated from a greater proportion of animals given thyroxine (Table 2). At 24, 48 and 72 hours virus in the ovary reached titres of $10^{-4\cdot5}$, 10^{-3} and $10^{-1\cdot7}$ respectively as against $10^{-2\cdot1}$, 10^{-2}, and 10^{-1} in controls.

With thyroxine, virus in the adrenals was increased to about the same extent as with ACTH, and once again mepacrine had very little influence in countering its growth. In both groups virus still persisted (over 10^{-2} with thyroxine) at 168 hours. The titres with thiouracil were rather lower than in controls and mepacrine sharply diminished them. The adrenals of the thyroxine-dosed mice were 2½ times the weight of the controls; in the groups receiving mepacrine in addition the increase in weight was twofold. Histologically the glands showed considerable change. The X-zone was lacking and the inner two-thirds of the cortical cells were swollen and excessively foamy. In one of 10 animals receiving virus in addition acute necrosis was present in the innermost layer adjacent to the medulla.

Thus differences emerge in the pattern of viral growth when this is stimulated by thyroxine instead of with one of the other hormones considered. The duration of the viraemia is not prolonged by thyroxine, the kidney is infective at an earlier stage, voluntary muscle as well as the heart is more frequently infective, and the inhibitory effect of mepacrine on viral growth in the adrenal is more drastically diminished than with oestradiol or cortisone and to about the same degree as with ACTH. Thiouracil has an action opposite to that of thyroxine.

DISCUSSION

For many years it has been postulated that a variety of constitutional factors play an important part in the outcome of viral infections, and nothing has emerged recently to contradict this belief. In the hormonal field relatively little has been studied in any detail, save the action of cortisone and ACTH. Abundant evidence exists that cortisone, but not usually ACTH, may influence viral infections to the detriment of the host. Earlier literature was adequately reviewed by Kass and Finland (1953) and the effect has been further demonstrated by Aronson and Shwartzman (1953, 1956), Shwartzman and Aronson (1953-4), Kass, Lundgren and Finland (1954), Boring, Angevine and Walker (1955), Iman and Hammon (1957), Teodoru (1957) and Starr and Pollard (1958). In the mammalian host the effect has often been associated with enhanced growth of virus, and detailed study of the data of at least three groups of workers (Kass *et al.*, 1954; Boring *et al.*, 1955; Teodoru, 1957) suggests that persistence of a high titre of virus rather than a greatly increased maximum titre is the pattern of viral growth on these occasions. This is certainly so in many tissues of cortisone-treated animals infected with the virus of equine encephalomyelitis; in this respect cortisone behaves similarly to oestradiol (Hurst *et al.*, 1960b), though, as described, several differences are apparent in the detailed behaviour of the two steroids. In addition both act to counter the suppressive effect of mepacrine on viral growth. Cortisone does not enhance viral growth in all situations e.g., in voluntary muscle and in lymphoid tissue, and Higginbotham, Stewart, Gebhardt and Samuels (1956) found it wholly devoid of influence on the growth of equine encephalomyelitis virus in the mouse brain. In another host (chick embryo), and under other conditions, cortisone may actually reduce the growth of viruses (Kilbourne, 1957 a, b; Siegel, 1958; Stewart, 1958), possibly because of its inhibitory action on growth and protein synthesis.

On the basis of a single experiment, it would seem probable that ACTH differs from both oestradiol and cortisone in its effect on equine encephalomyelitis and, except in the adrenals, it seems to have relatively little influence on the suppression of viral growth by mepacrine.

Our results with thyroxine and thiouracil are diametrically opposed to those of Holtman (1946) with intracerebrally injected poliomyelitis virus in the mouse. He obtained earlier paralysis with thiouracil and considerable prolongation of the incubation period with thyroid extract or thyroprotein; there is no indication in his article that thiouracil and thyroid extract were administered for some days prior to infection to ensure that an altered metabolic state should exist from the moment of introducing virus. With various doses of crystalline thyroxine Gollan (1948) could demonstrate no effect on intraperitoneally administered MM virus. Weiss, Moulder and Itatani (1952) lowered survival time by administering thyroxine to mice infected by inhalation of murine pneumonitis virus, and observed longer survival in mice in which the thyroid had been eliminated by a dose of radioactive iodine. They were unable to detect increased multiplication of virus in the presence of the hormone, and, in tissue-cultures, Eaton, Adler, Bond and Scala

(1956) observed only slight effects of thyroxine on the growth of influenza virus, and then not always in the same direction.

Our own experiments with equine encephalomyelitis demonstrate clear enhancement of viral growth in mice treated with thyroxine. In most tissues this increase takes the form of considerable exaggeration of the normal rise in viral titres, without the prolongation of high titres seen with oestradiol and cortisone. The whole disease is accelerated, so that the majority of deaths occur at a much earlier period than with the steroid hormones, while thiouracil tends to move the cumulative death-curve in the opposite direction. Although with oestradiol, cortisone and thyroxine, isolations of virus from cardiac muscle are favoured, and with thyroxine from voluntary muscle also, none of the mice showed histologically the florid myositis produced under analogous circumstances in cortisone-prepared hamsters by MEF_1 poliomyelitis virus (Aronson and Shwartzman, 1953). Combined with oestradiol, stilboestrol and thyroxine, however, the virus of equine encephalomyelitis is able sometimes to produce necrosis in the already considerably damaged adrenal cortex.

While in the experiments we have detailed the dosage of the various hormones was relatively very large, it is clear that hormonal imbalance may sharply modify infection with the virus of equine encephalomyelitis and may profoundly influence therapy with a chemotherapeutic agent. All the hormones having this property act to increase growth of virus and to diminish the suppressive action of mepacrine, and, by producing more severe or more prolonged viraemia, render infection of the central nervous system and death more certain (Hurst, 1950). Whether their other, and differing, actions affect this particular issue the experiments do not disclose. It is unlikely that antibodies have any relation to the course of events, which evolve at a stage prior to that at which antibodies can be convincingly demonstrated; moreover, our own limited observations on this point have failed to reveal clear differences between the variously treated groups in the speed with which antibodies are formed or in their titres when they first appear.

CONCLUSIONS

Like oestradiol and stilboestrol, and unlike testosterone and progesterone, cortisone, ACTH and thyroxine all stimulate the growth of the virus of equine encephalomyelitis in the mouse. They abolish the differential mortality which normally exists, in favour of the female, between the sexes. They tend to counteract the prophylactic effect of mepacrine against the infection. The patterns of viral growth with the various hormones are described in some detail and are shown to differ appreciably. Thiouracil acts in a direction opposite to that of thyroxine.

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GLOMUS TUMOUR IN A DOG

By

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CASE HISTORY

A male dachshund, 10 years of age, was presented for examination in October, 1955. The dog appeared to be in pain, moving only reluctantly and holding its left fore foot off the ground. There was a history that in the previous week there had been profuse bleeding from the toes. The site was extremely painful and the animal resented interference with it. There was no mention of an accident.

In order properly to examine the lesion, 2 ml. Demerol were injected intravenously. The animal was in good condition. The visible mucosae were of normal colour. The rectal temperature was 39·2°C. The frequency of respiratory movements and heart beats could not be determined, due to the great excitement of the animal. The retropharyngeal and popliteal lymph nodes were palpable and mobile, and the size of an olive.

Examination of the left fore foot showed a nodular lesion beneath the digital cushion of the second toe. The surface showed signs of haemorrhage. The nodule was soft and was adherent to the skin but not to the deep layers. Surgical removal was easy and the post-operative course was satisfactory. The symptoms had disappeared immediately after the operation and since then the dog has been in perfect health.

Gross Description

The specimen was a rounded fairly firm nodule, 1·5 cm. in diameter and reddish in colour; the surface showed a small area of haemorrhagic appearance. The surrounding area appeared to consist of the remains of epidermal tissue, showing slight ulceration and some dried blood. The rest of the lump was enclosed in areolar connective tissue. On section, a reddish pink surface with a small haemorrhagic area near one of its borders was disclosed. The appearance was that of a solid glandular tumour.

Microscopic Description

Fragments of the tumour were fixed in 10 per cent neutral formalin, embedded in paraffin and stained by haematoxylin and eosin, van Gieson, Mallory, Junqueira and Martins, (mod. 1947), Weigert and acid orcein, Heidenhain's ferric haematoxylin, Bielschowski, Foot-Wilder, and Masson's trichrome stains.

Microscopic examination showed compact cell groups placed specially around blood vessels and surrounded by a network of very delicate connective fibrils which were easily stained by selective methods. The cells also appeared in strings of 2 or 3 rows along the blood vessels which were typically of Masson's type II. The cells were light, rounded or polyhedral in shape, and contained abundant cytoplasm, usually acidophilic, but at times with a basophilic tendency. Some had 2 or 3 nuclei. Due to their disposition and shape, these cells are called epithelioid or glomus cells.

The nuclei were large and rounded, some being kidney shaped and others irregular. Most of them contained little chromatin and had a vesicular appearance. A few were pycnotic. One or two karyosomes were apparent and upon careful examination various phases of mitosis could be seen. Among these epithelioid cells a few spindle-shaped cells, having long nuclei with rounded ends could be observed, but selective stains failed to demonstrate myofibrils in these cells. In some sections the remains of epidermis were found, covered by a crust but the periphery of the tumour showed a marked purulent inflammatory reaction, with congestion and small foci of necrotic tissue.

Non-medullated nerve fibrils were embedded in the tumour mass, but there were no myelinated fibres, even at the periphery. The stroma consisted of vascular connective tissue and was poorly developed. There was no proper capsule.

Silver impregnation methods revealed a delicate wide-mesh reticulum, frequently surrounding the blood vessels and extending throughout the tumour. The neoplastic tissue contained no elastic fibres, although a few were encountered near blood vessels.

The growth was diagnosed as a glomus tumour of epithelioid type.

DISCUSSION

The case is believed to be the first glomus tumour described in a dog, as the one published by Trein (1948) resembles a Sticker tumour on account of its location in the vagina, the absence of characteristic symptoms and the photographs shown.

The symptoms observed in the present case are exactly similar to those described in human medicine. These tumours were first noticed because they caused great pain and were originally recognised by Bisset (1792). Wood (1812) reviewed all tumours of the kind then recorded in the literature and referred to them as "subcutaneous painful tubercles." Subsequently they were looked upon as angiosarcomas, angiomas, epitheliomas, neuromas of the Vater-Paccini corpuscles, periepitheliomas, etc., until finally Barré and Masson (1924) demonstrated the presence of three elements: nervous, muscular and vascular, thus defining these growths as angio-neuromyomas. Masson (1924) studying the neuro-myo-arterial glomus found that it was a special type of arterio-venous anastomosis which was the origin of tumours which he called glomus tumours. Bailey (1935) suggested the name of glomangiomas. Martorell (1940) published a monograph mentioning 124 glomus tumours, including the ones originally referred to by Bisset. In Brazil, glomus tumours were described in man by Gonzales Torres (1945), Abdala and de Mello (1950), Lopes Jr., Long and Marchon (1953), Becker (1953) and Pondê and Andrade (1955).

The present case, in a dog, shows an anatomopathological picture identical with that found in human pathology, besides having the same symptoms and location. The digital extremity is the most common location for this type of blastoma, although some extradigital glomangiomas have been reported by Prodanoff (1927), Masson and Gery (1927), Mason and Weil (1934), and Bailey (1935). They are, as a rule, benign, although, as stated by Becker (1953), they may occasionally produce metastases and recur. According to the predominance of either the nervous, vascular or cellular elements the following classification has been proposed for this tumour: neuromatous, angiomatous or epithelioid.

CONCLUSIONS

A record of a case of glomus tumour located on a digital extremity of a dog which showed symptoms of acute pain and lameness. From the gross and microscopic picture of the lesion a diagnosis of glomangioma of epythelioid type is made.

The location, symptoms and the gross and histological appearance are identical with those described in human pathology.

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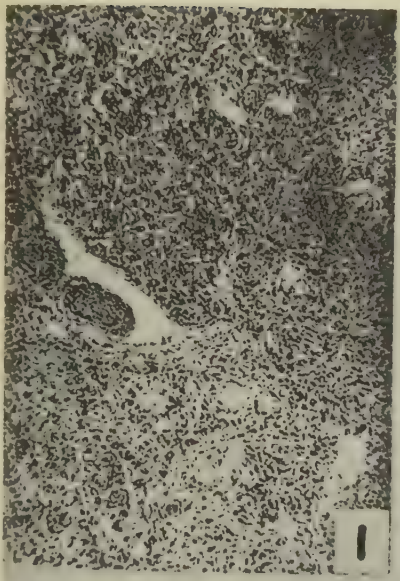

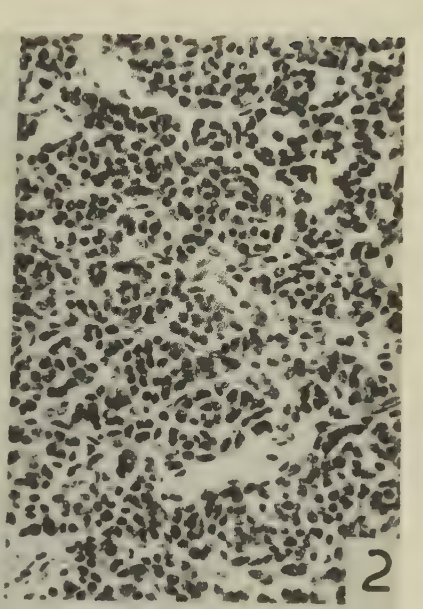

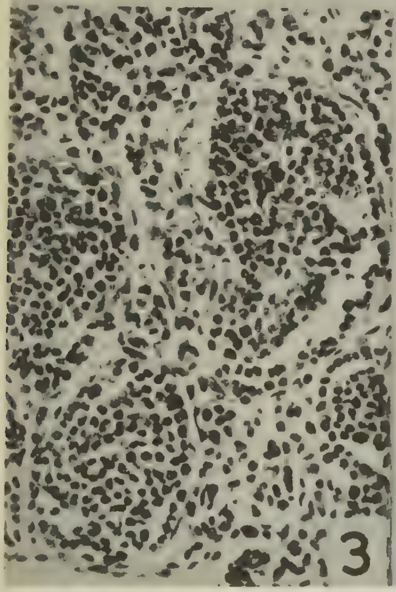

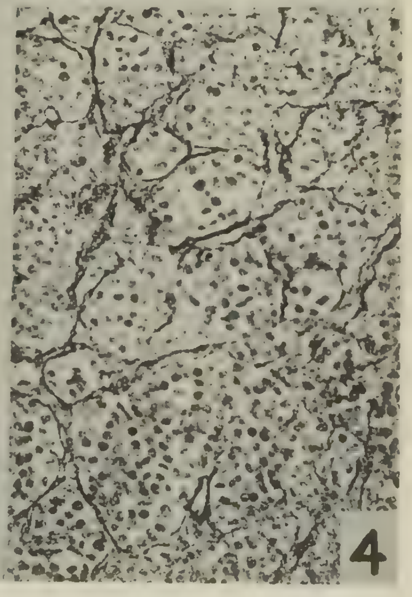

Fig. 1. Glomangioma. H.E. 100×.

Fig. 2. Glomic tumour showing cells arranged in cords. H.E. 300 ×.

Fig. 3. Glomic tumour showing cells arranged in groups surrounded by connective tissue. Masson's trichrome 300 ×.

Fig. 4. Glomic tumour showing the reticulum. Foot-Wilder, 200 ×.

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DISTURBANCE OF RESPIRATION OF CALVES CAUSED BY PNEUMONIA DUE TO *DICTYOCAULUS VIVIPARUS*

The Changes Taking Place in Respiratory Rate, Ventilation Rate, Plasma CO_2 Content and Plasma pH

By

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INTRODUCTION

A pneumonic process affecting the lung may cause interference with gaseous exchange by a thickening of the exchanging surface, the alveolar wall, due to hyalinisation or epithelialisation, by inflammatory exudation interfering with the passage of air in and out of the lung and by actual destruction of lung tissue. The pathological changes taking place in the lungs of cattle as a result of pneumonia have been extensively studied by Jarrett (1956) and with particular reference to bovine parasitic bronchitis by Jarrett, McIntyre and Urquhart (1957). The disturbance of respiratory rate of calves with pneumonia caused by *Dictyocaulus viviparus* has been followed by Jarrett, Jennings, McIntyre, Mulligan and Urquhart (1955) and by Jarrett, Jennings, McIntyre, Mulligan and Urquhart (1957), and has been used by them to assess the severity of the pneumonic lesion. Sampson and Hayden (1935) have reported that they could detect no change in the carbon dioxide capacity of the plasma of four cows affected with pneumonia but did not state how severely affected their cases were.

Observations were made on the changes in respiratory rate, ventilation rate, plasma CO_2 content and plasma pH of calves infected with *D. viviparus* and showing clinical evidence of pneumonia. A number of experiments were carried out to determine the plasma changes in pneumonia and later to attempt to correlate these changes with the disturbances of respiration.

METHODS

The animals were castrated male calves bought in the open market when 3 to 10 days old. They were housed separately in metal pens and fed Ostermilk no. 2*, 1 lb. to a gallon of water. They received six to eight pints daily for the first three weeks and were also offered calf weaner pencils and hay to nibble at after two weeks. At the beginning of the fourth week they received one-seventh less dried milk but the water intake was maintained at six to eight pints. The gradual reduction of the dried milk was continued on a weekly basis so that at eight weeks the dried milk was completely stopped. At this time the calves were eating several pounds of hay and calf weaner pencils per day. At 10 to 12 weeks the calf weaner pencils were replaced by calf rearing nuts of a lower protein value.

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Most calves were dosed with infective larvae at 6 to 8 weeks of age but in one experiment some were exposed to infected pasture instead. Calves described as normal were not infected with *D. viviparus* but sometimes had some slight disturbance of respiration due to cuffing pneumonia, but in no case was the respiratory rate in excess of 40 per minute. Calves described as pneumonic had a well-established clinically obvious pneumonia with increased respiratory rates usually in excess of 50 per minute and often much higher.

The ventilation rate was measured by means of a face mask leading to three-quarter inch diameter uni-directional valves of the diaphragm type which were connected on the expiratory side to a Parkinson-Cowan gas meter. Figure 1 shows a calf having its ventilation rate measured. The respiratory rate was measured by counting the clicking sounds of the uni-directional valves. Ventilation and respiratory rates were measured for periods of 10 minutes and the mean value per minute was calculated.

Blood samples for the determination of the plasma CO_2 content and the plasma pH were removed from either the jugular vein or the brachial artery (Fisher, 1956). The samples were collected without stasis into heparinised centrifuge tubes under a layer of mineral oil so that no exposure to the atmosphere took place. They were immediately centrifuged and the plasma pH and the plasma CO_2 content determined. The plasma pH was measured by an adaptation of a method of Van Slyke (Fisher, 1959a) and the plasma CO_2 content was determined by the method of Conway (1957) adapted to suit the conditions prevailing (Fisher, 1959b).

RESULTS

Experiment 1

The plasma carbon dioxide content of arterial blood was determined in two groups of calves of about eight weeks of age. Blood samples taken in the morning after the calves had been fed, were immediately centrifuged and the plasma carbon dioxide content determined. Sampling was carried out on different days so that some calves were sampled more than once. The mean value for the pneumonic calves was significantly higher than for the normal calves (Table 1) indicating that carbon dioxide retention had occurred in the former.

Experiment 2

The arterial plasma pH was determined on two groups of calves of about nine weeks of age. The pneumonic calves had been infected three weeks previously with 4,000 *D. viviparus* larvae and were well established cases of severe parasitic pneumonia with greatly increased respiratory rates. Table 2 gives the results obtained. A lower mean value was found for the plasma pH of the pneumonic calves but the difference was not significant.

Experiment 3

Both the arterial plasma CO_2 content and the pH were measured simultaneously in five normal calves and in 19 severely affected pneumonic calves all about 10 weeks old. The results (Table 3) confirmed that parasitic pneumonia caused a carbon dioxide retention with a significantly higher carbon dioxide content of the plasma of the pneumonic calves but no significant difference in plasma pH.

TABLE 1

CARBON DIOXIDE CONTENT OF THE PLASMA OF NORMAL AND PNEUMONIC CALVES

	Pneumonic calves		*Normal calves*	
Date	*Calf No.*	*CO_2 content (millimols /litre)*	*Calf no.*	*CO_2 content (millimols /litre)*
4/6	33	32·9	18	30·2
	34	32·4	19	28·4
	35	32·8		
	36	32·4		
	37	34·6		
7/6	30	33·7	15	31·1
	31	33·7	16	31·9
	32	30·5	17	31·0
	33	35·6	18	31·4
	34	31·5	19	31·0
11/6	35	32·0	40	29·5
	36	31·7	41	29·6
	37	31·4	42	27·4
	38	33·7	43	28·8
	39	31·0	44	25·2
12/6	30	31·9	16	28·6
	31	28·7	17	25·6
	33	30·5	18	28·8
	34	29·2	19	24·3
Mean values	(19)	32·6 ± 1·8	(16)	28·9 ± 2·3

Experiment 4

In this experiment a direct comparison was made by means of single determinations of respiratory rate, ventilation rate, venous plasma pH and the plasma CO_2 content of jugular blood of two groups of calves of about 10 weeks of age. One group was infected for three weeks by grazing a pasture heavily contaminated with *D. viviparus* larvae while the other uninfected group had been reared indoors in isolation. Table 4 gives the results obtained. Marked differences were observed. The carbon dioxide content of the venous plasma of the pneumonic calves was significantly higher than in the uninfected calves but apart from calf (e) in the pneumonic group the plasma pH was not outside the range of normality previously determined (Fisher, 1959a). By comparison with the uninfected calves, there was an increase in both ventilation and respiratory rate, but as the increase in the latter was much the larger the tidal air was diminished and so respiration was shallower.

TABLE 2

PLASMA pH OF NORMAL AND PNEUMONIC CALVES

Normal calves		*Pneumonic calves*	
Calf no.	*pH*	*Calf no.*	*pH*
9722	7·47	9836	7·38
9723	7·44	9831	7·44
9718	7·42	9842	7·42
9725	7·44	9834	7·44
9726	7·45	9833	7·40
9727	7·47	9818	7·39
9730	7·44	9828	7·38
9731	7·40	9821	7·44
9740	7·47	9819	7·38
9802	7·42	9816	7·42
9739	7·38	9914	7·36
9736	7·42	9817	7·40
9512	7·38	GL17	7·39
Total = 13	Mean = 7·43 ± 0·04	Total = 13	Mean = 7·40 ± 0·04

TABLE 3

PLASMA pH AND PLASMA CO_2 CONTENTS OF NORMAL AND PNEUMONIC CALVES

	Normal calves			*Pneumonic calves*	
Calf no.	*pH*	*CO_2 content (millimoles/litre)*	*Calf no.*	*pH*	*CO_2 content (millimoles/litre)*
31	7·38	27·4	4	7·42	27·8
32	7·47	27·8	5	7·44	29·1
33	7·44	27·9	GL17	7·42	31·2
34	7·36	28·8	GL15	7·38	26·4
931	7·42	27·6	62	7·40	30·4
Total 5	Mean 7·41 ± 0·04	27·9 ± 0·54	61	7·42	30·1
			55	7·33	32·0
			56	7·34	32·6
			57	7·36	32·4
			58	7·40	29·1
			59	7·38	33·5
			60	7·36	30·5
			37	7·38	29·8
			38	7·40	28·3
			39	7·36	28·8
			49	7·36	26·8
			932	7·38	29·0
			933	7·46	29·9
			763	7·30	28·3
			Total 19	Mean 7·38 ± 0·04	29·8 ± 2

Experiment 5

Five calves were infected with 4,000 *D. viviparus* larvae and one week later the respiratory and ventilation rates and the venous plasma pH and carbon dioxide content were measured and were repeated at weekly intervals for six weeks. The results for each calf are given in Table 5 and the mean values are summarised in Table 6.

Calf 99 responded to the infection with an increase in plasma CO_2 and a tendency for the plasma pH to fall. The respiratory rate increased to a greater extent than the ventilation rate so that the tidal air fell, indicating shallower respiration. Calf 100 showed more marked changes in all the measurements. Calves 97 and 98 also showed great changes in plasma CO_2 content but no marked changes in plasma pH. The changes in plasma CO_2 in calf 55 were quite marked and somewhat erratic. Except for the last determination, no great changes in plasma pH were observed. This final low pH was for a blood sample which had a particularly low plasma CO_2 content and these two low values together may indicate a disturbance other than respiratory. The changes in respiratory rate were less marked than in the other four calves. The mean values for the five calves show again that plasma pH was little affected but that plasma CO_2 content rose significantly. Ventilation rates increased to a much less extent than respiratory rates; in consequence tidal air fell and respiration was very much shallower.

TABLE 4

PLASMA pH, PLASMA CO_2 CONTENT, VENTILATION AND RESPIRATORY RATES AND TIDAL AIR OF NORMAL AND PNEUMONIC CALVES

Calves	*CO_2 (millimoles /litre)*	*pH*	*Ventilation (litres/min.)*	*Respiratory rate/min.*	*Tidal air (ml.)*
Normal					
a	28·8	7·40	28	41	680
b	31·3	7·38	22	35	620
c	29·4	7·35	26	30	860
d	29·1	7·38	24	30	800
Mean	29·8	7·38	25	34	740
Infected					
e	37·6	7·26	33	75	400
f	31·8	7·20	30	62	480
g	34·5	7·35	40	80	500
h	35·1	7·40	38	87	430
i	32·3	7·35	45	82	540
Mean	34·3	7·33	37	77	470

TABLE 5

CHANGES IN PLASMA CO_2 CONTENT, PLASMA pH, VENTILATION AND RESPIRATORY RATES AND TIDAL AIR DURING PNEUMONIA

Date	*CO_2 (millimoles /litre)*	*pH*	*Ventilation (litres/min.)*	*Respiratory rate/min.*	*Tidal air (ml.)*
			Calf 99		
12/9	28·8	7·40	28	41	680
19/9	28·6	7·38	24	50	480
26/9	31·5	7·44	27	75	360
3/10	30·6	7·38	33	57	580
10/10	34·6	7·37	29	50	580
21/10	31·6	7·33	37	69	540
			Calf 100		
12/9	31·3	7·38	22	35	630
19/9	31·3	7·38	31	93	330
26/9	36·2	7·39	24	78	308
3/10	35·8	7·38	29	97	300
10/10	37·9	7·34	30	89	337
21/10	35·1	7·32	37	80	462
			Calf 97		
12/9	29·4	7·35	26	30	870
19/9	26·9	7·39	18	32	560
26/9	34·0	7·44	28	52	540
3/10	33·4	7·40	26	57	460
10/10	34·6	7·38	18	43	420
21/10	36·3	7·36	36	73	490
			Calf 98		
12/9	30·6	7·35	12	44	272
19/9	28·4	7·39	15	60	250
26/9	30·5	7·41	18	69	260
3/10	32·5	7·46	18	66	272
10/10	33·5	7·39	16	78	205
21/10	35·6	7·38	25	116	215
			Calf 55		
12/9	29·1	7·38	24	30	800
19/9	27	7·36	25	47	530
26/9	36·7	7·44	30	55	545
3/10	31	7·38	32	52	615
10/10	34·3	7·38	32	50	640
21/10	26·3	7·28	31	55	563

TABLE 6

MEAN VALUES FOR ALL 5 CALVES DURING PNEUMONIA

Date	*CO_2 (millimoles /litre)*	*pH*	*Ventilation (litres/min.)*	*Respiratory rate/min.*	*Tidal air (ml.)*
12/9	29·8	7·37	20	36	555
19/9	28·4	7·38	23	56	410
26/9	33·7	7·42	25	66	378
3/10	32·6	7·39	26	66	393
10/10	34·9	7·37	25	62	403
21/10	32·9	7·33	33	79	418

DISCUSSION

Calves affected with parasitic pneumonia showed an increase in both ventilation rate and respiratory rate but as the increase in the latter was much greater the respiration became shallower and so less efficient. To obtain an adequate gaseous exchange the calves had therefore to use more energy in order to breathe.

In the majority of pneumonic calves the plasma CO_2 content was significantly higher than in the normal calves, although not all the values lay outside the extremes for non-pneumonic calves (Fisher, 1959c). With the exception of two pH values the plasma pH showed no marked change from previously determined normal values (Fisher, 1959a). A normal plasma pH, together with a raised plasma CO_2 and hence a raised plasma bicarbonate concentration, indicated a compensated respiratory acidosis, the compensation being possibly renal in origin.

The increased respiratory response in the pneumonic calves must have been due to a continuous stimulus to respiration as a result of the pneumonic process. The stimulus was not a change in plasma pH as this did not alter to any significant extent. The plasma CO_2 content, however, was significantly raised and may well have been the main stimulus but other factors may also have been responsible, such as the pathological cellular changes in the lungs themselves and a reduced oxygen content of arterial blood, which was suspected from the darkened appearance of this blood when taken from pneumonic calves. No determinations of the oxygen content were made. Further work is required to elucidate the importance of the various factors.

CONCLUSIONS

Measurements of respiratory rate, ventilation rate, plasma pH and plasma CO_2 content have been made on calves with pneumonia caused by *D. viviparus*.

A greatly increased respiratory rate was accompanied by a smaller increase in ventilation rate, indicating shallower respiration.

No marked decrease from normal was found in the plasma pH but the plasma CO_2 content was significantly above normal, indicating a compensated respiratory acidosis.

ACKNOWLEDGMENT

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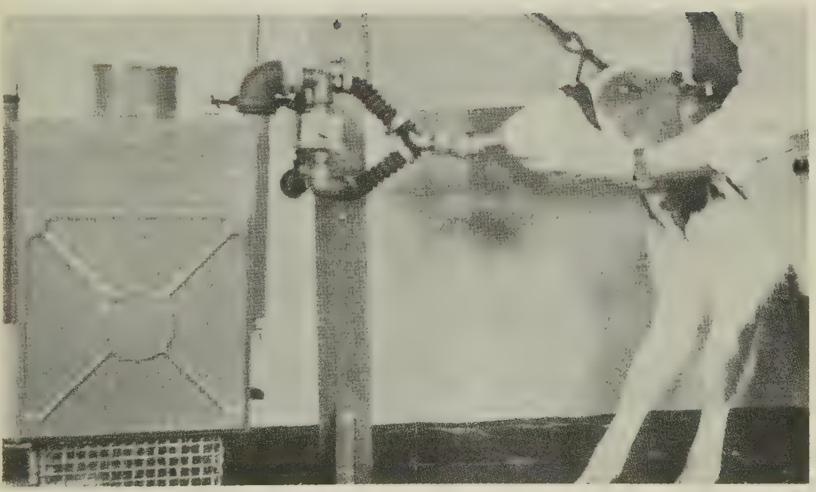
Fig. 1



Measurement of the ventilation rate of a calf.

J. Comp. Path. 1960. Vol. 70.

PROPHYLAXIS AGAINST REPEATED ARTIFICIAL CHALLENGE WITH *TRYPANOSOMA CONGOLENSE*

A Comparative Trial of Antrycide Pro-Salt and Prothidium Bromide

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INTRODUCTION

The extensive conditions under which cattle are commonly kept in Africa and the handicap of poor communications combine to make frequent treatment of individual animals both uneconomical and impracticable. These factors limit the value of even the most effective curative drug for the control of trypanosomiasis in areas where the disease is enzootic, since re-infection soon after treatment, either by tsetse flies or mechanical transmission, is always probable. A drug conferring protection for a reasonable period would therefore mark a valuable advance in the control of this disease. Ideally, a single injection of such a compound would confer complete protection against trypanosomiasis for at least twelve months, but a drug giving six months protection would be of value in those regions where cattle can be effectively intercepted and treated on their migrations between wet and dry season grazing areas.

The first trypanocidal drug to show clear promise of prophylactic properties in cattle was Antrycide Pro-salt† and the numerous reports on the use of the drug for this purpose have been reviewed by Davey (1957). In the Sudan, cattle have now been kept successfully under light to moderate tsetse challenge for over six years, using bi-monthly injections of Antrycide Pro-salt at a dosage rate of 12 mg./kg. body weight. Because of the dangers of a resistant strain developing if infection occurs when the concentration of drug in the body is waning, this type of protection is only suitable for cattle under effective control, i.e. where regular treatment and proper disposal of animals leaving the herd can be ensured.

Ethidium Bromide‡ is of limited value for prophylactic purposes, although now well established as an effective drug for the curative treatment of *Trypanosoma congolense* and *T. vivax* infections in cattle. Leach, Karib, Ford and Wilmshurst (1955) found that treatment at the rate of 1 mg./kg., the recommended curative dose, protected only a proportion of their groups of cattle for four weeks against artificial challenge with *T. congolense* and for six weeks against *T. vivax*. Dosage at 5 mg./kg., only slightly prolonged the protection periods and resulted in a number of cattle suffering from drug toxicity.

Watkins and Woolfe (1956) reported that two pyrimidyl-substituted

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[†] Imperial Chemical (Pharmaceuticals) Ltd. (Quinapyramine Prophylactic B.Vet.C.).

[‡] Messrs. Boots Pure Drug Co., Ltd. (Homidium Bromide, B.Vet.C.).

phenanthridinium drugs developed by them had marked prophylactic qualities in mice and one of these, RD 2801, 2-amino-7-(2′-amino-1′:6′-dimethylpyrimidyl-4′-amino)-9-p-aminophenyl-10-methylphenanthridinium dibromide, named "Prothidium"*, showed considerable promise in preliminary field trials in cattle, against both *T. congolense* and *T. vivax,* in Tanganyika and Nigeria. Thus Robson and Milne (1957) reported that Prothidium, administered at 2 mg./kg. body weight, protected groups of six cattle against single syringe challenge with *T. congolense* two, four and six months after treatment. Three cattle from the first two of these groups were re-challenged seven months after drug treatment and one showed a persistent infection. No further challenges were recorded.

The experiment described in this paper was designed to compare the prophylactic qualities of Antrycide Pro-salt (old formulation) and Prothidium Bromide in the face of repeated artificial challenge with *T. congolense.*

MATERIALS AND METHODS

The animals under experiment were kept at the Veterinary Laboratory, Malakal, Upper Nile Province, a tsetse-free area where mechanical transmission of trypanosomiasis occurs, particularly during the rains (May to October). In September, 1956, thirty-six animals from a random group of young Shorthorn Zebu cattle were inoculated subcutaneously with Antrycide Pro-salt, prepared and administered in accordance with the manufacturer's instructions; the site of injection was on the side of the thorax, slightly above and behind the elbow. Cattle between 150 and 200 kg. body weight received 10 ml. per head and those between 200 and 350 kg. were given 15 ml. per head. Thirty-seven similar cattle received 2 mg./kg. Prothidium Bromide in a 2 per cent aqueous solution, twenty being inoculated subcutaneously, in the same site as that used for the Antrycide injections, and seventeen intramuscularly in the buttocks. A subsidiary group of six cattle was inoculated intramuscularly with Prothidium Bromide, at a dosage rate of 5 mg./kg., the injection being divided equally between both buttocks.

All the cattle were grazed together and herded at night in the laboratory compound. They were identified by horn branding. Two months after treatment, seven cattle from each of the principal groups, together with three untreated controls, were challenged by the subcutaneous inoculation of 5 ml. citrated blood from an ox naturally infected with *T. congolense* and showing a marked parasitaemia.

Seven fresh cattle from each principal group and three untreated controls were similarly challenged at about monthly intervals thereafter. At the same time, all treated cattle in previous challenge groups were re-challenged. On the morning when a challenge was due, wet films were examined from the three cattle used as controls in the previous challenge and blood for the new challenge was taken from the animal with the heaviest parasitaemia. When all cattle in the two principal groups had been challenged, regular monthly re-challenges were continued until persistent breakdowns occurred.

Since insufficient staff was available for daily slide examinations, two thick blood films were taken from all animals after challenge on three consecutive days each week, either until the end of the experiment or until persistent infections occurred. The blood films were stained by a modification of Field's method (Neave, 1952) and about 200 fields were examined on each slide, using the 1/12″ oil immersion objective. When a persistent breakdown occurred, the animal was treated with 0·25 g. Ethidium Bromide in 2 per cent aqueous solution inoculated intramuscularly into the buttocks, and was discharged from the experiment.

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* Messrs. Boots Pure Drug Co., Ltd.

RESULTS

Systemic Reactions

The cattle were kept under observation immediately after drug treatment and no reactions were observed among those injected with Prothidium Bromide. Four of the cattle inoculated with Antrycide Pro-salt started panting, became restless and sought a shady tree or bush, lying down for about ten minutes before resuming grazing. All the other cattle in this group behaved normally. The animals remained in good condition throughout the experiment.

Local Reactions

A week after inoculation, nine cattle which received Prothidium Bromide subcutaneously had swellings at the site of injection, ranging from 2″ × 3″ to 4″ × 8″. Two cattle inoculated intramuscularly (one received 2 mg./kg. and the other 5 mg./kg.) showed swellings measuring about 2″ × 3″. All these local reactions had decreased after another week and disappeared within three weeks of treatment. There was no necrosis or sloughing at the site of injection. The cattle inoculated with Antrycide Pro-salt showed the customary small swelling; this persisted for over two months in most of the bulls without sloughing. No lameness resulted from these injections.

Deaths

Three of the six beasts inoculated with 5 mg./kg. Prothidium Bromide died fifty-five, sixty and sixty-three days respectively after treatment. Detailed post mortem examinations could not be performed, due to the absence of both authors from Malakal at the time, but the Veterinary Assistant in charge reported that, after showing inappetence for two days, each animal became recumbent and died rapidly. The drug was found to be widely distributed in the muscles surrounding the injection site. These uniform symptoms suggested that the deaths were due to drug toxicity, and subsequent observations in both the Sudan (E.A.K.) and Nigeria (T.M.L.) confirm that a substantial proportion of cattle treated with Prothidium Bromide at this dosage rate will die between six and twelve weeks later, showing characteristic degenerative changes in the liver on microscopic examination. A fourth animal in this group died 127 days after treatment. At autopsy it was found to be severely affected with liver fluke (*Fasciola gigantica*). The two remaining animals in this group survived to the conclusion of the observation period. All the other treated animals with the exception of three which died accidentally, remained in good condition until the end of the experiment. One of the three had shown a persistent infection with trypanosomes from the 307th day after treatment.

Blood Examinations

Table 1 summarises the pattern of challenge inoculations and blood film examinations.

Occurrence of persistent infections. The earliest persistent infection occurred in ox 45, treated with Prothidium Bromide and included in the third challenge group. Trypanosomes were first seen on 20th April, 1957, 215 days post-treatment and eight days after this group received its fourth challenge. The first persistent infection in an Antrycide Pro-salt treated ox was observed in 27, in the first challenge group. Trypanosomes were initially seen on 3rd June, 1957, 258 days post-treatment and twenty-four days after this group received its seventh challenge.

Table 2 contains details of all persistent infections observed. Due to pressure of other work, examination of the slides taken in the fortieth, forty-first, forty-second and forty-seventh weeks post-treatment had to be deferred and, when this was undertaken later, it was found that the films had decomposed. However, as may be seen from Table 2, an additional ten animals treated with Prothidium Bromide and a further two treated with Antrycide Pro-salt had developed persistent infections before the end of the forty-ninth week. The observation period was concluded on 30th September, 1957, fifty-four weeks after treatment. At the end of the experiment, nineteen cattle treated with Prothidium Bromide and eight treated with Antrycide Pro-salt had shown persistent infections, while eighteen and twenty-six respectively remained negative throughout. Under the conditions of this experiment, the duration of protection seems to bear no relationship to the number of challenges received.

Occurrence of transient infections. As will be seen in Table 1, trypanosomes were noted in the blood of each of five cattle, three out of thirty-seven treated with Prothidium Bromide and two out of thirty-six with Antrycide Pro-salt, on one day only many weeks before persistent infections occurred in these animals. Usually only one blood film was examined of the two prepared from every animal each day films were taken, but, when parasites were seen, the second film was also examined. Trypanosomes were always seen in both films—thus reducing to insignificance the possibility of a false positive due to the necessity of washing and using slides more than once. Table 3 gives details of these transient infections and shows the periods before persistent infections were noted in the same animals. The significance of the former will be considered in the Discussion.

Live Weights

The cattle were weighed at the time of treatment and again in

TABLE 1

SUMMARY OF CHALLENGES AND RESULTS OF BLOOD FILM EXAMINATIONS

Challenge Group	*Drug*	*Completed weeks after drug treatment*																													
		23	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	*41*	*42*	*43*	*44*	*45*	*46*	*47*	*48*	*49*	*50*	*51*	*52*	*53*	*54*
First	Prothidium Bromide																						1				1	1					
	Antrycide Pro-salt														1													1					
Second	Prothidium Bromide																											1	1		1		
	Antrycide Pro-salt					T																											
Third	Prothidium Bromide							T	1													1		1			1				1		
	Antrycide Pro-salt																					2											
Fourth	Prothidium Bromide					T																1	1					2					
	Antrycide Pro-salt																											1	1				
Fifth	Prothidium Bromide							T															1				1	1					
	Antrycide Pro-salt												T																	1	1		
Sixth	Prothidium Bromide																								1								
	Antrycide Pro-salt																																
Challenge Dates†						16.3.57			12.4.57				10.5.57				5.6.57					6.7.57				5.8.57				2.9.57			

Key: T = Transient infection. Trypansomes seen in both blood films from one bull on a single day.

1, 2 = Persistent infections occurred in one or two bulls in this group during the week in question. The affected cattle were treated with Ethidium Bromide and discharged from the experiment.

The first to fifth challenge groups all contained seven cattle treated with each drug. The sixth group comprised two treated with Prothidium Bromide and one with Antrycide Pro-salt. Cattle receiving Prothidium Bromide were treated on 17.9.56 and those having Antrycide Pro-salt on 18.9.56.

† = Dates of the earlier challenges not shown in the Table were 23.11.56, 21.12.56, 18.1.57 and 16.2.57.

TABLE 2

SUMMARY OF PERSISTENT INFECTIONS OCCURRING BETWEEN TREATMENT AND CONCLUSION OF THE OBSERVATION PERIOD (54 WEEKS)

Weeks post-treatment	*30*	*37* *	*43*	*44*	*45*	*46* *	*48*	*49*	*50*	*51*	*52*	*Total break-downs*	*Died before infected*	*Remained negative*	*Total in group*
Prothidium Bromide ..	1	—	2	3	1	1	3	5	1	—	2	18	—	19	37
Challenges Received ..	4		6, 7	5, 6, 9	7	4	6, 8, 10	6, 7, 9, 10	9		9, 10				
Antrycide Pro-salt ..	—	1	2		—		—	2	1	1	1	8	2	26	36
Challenges Received ..		7	7					7, 10	7	7	7				

*Slides taken during the 40th, 41st, 42nd and 47th weeks were not examined.

November, 1957. The initial average weight of the thirty-two animals treated with Prothidium Bromide and which survived to the end of the observation period was 195·3 kg. The corresponding figure for the thirty-three head which received Antrycide Pro-salt was 202·8 kg. At the conclusion of the experiment, the average weights of these groups were 227·9 kg. and 254·1 kg. respectively. Thus the average live weight gain of the cattle treated with Prothidium Bromide was 32·6 kg. and that of the Antrycide Pro-salt group was 51·3 kg. over the same period. This difference is significant at the one per cent level.

TABLE 3

OCCURRENCE OF TRANSIENT INFECTIONS

Bull No.	*Challenge Group*	*Drug*	*Transient infections*		*Persistent infections*	
			Days post-treatment	*Number of challenges*	*Days post-treatment*	*Number of challenges*
33	4	Prothidium	189	2	348	7
10	2	Antrycide	194	4	403	11
3	3	Prothidium	201	3	391	10
38	5	Prothidium	203	1	348	6
49	5	Antrycide	237	3	362	7

The final column shows the total number of challenges between treatment and persistent breakdown.

Reactions of Challenge Controls

Altogether thirty-three cattle were inoculated as controls at the eleven challenges. Apart from the three cattle used in the first challenge, which showed a parasitaemia when first examined sixteen days after inoculation, all these cattle had positive blood films within eight days of inoculation. When two of the three control animals in the succeeding challenge group had positive blood films, the three cattle in the previous group were treated with 0·25 g. Ethidium Bromide, with the exception of the animals mentioned in the next paragraph. These curative inoculations were made twenty-eight to fifty-six days after challenge and all the controls survived after treatment to the end of the experiment.

The three controls in group five were intentionally left untreated to observe the virulence of the organism. Two of these animals died sixty-one and eighty-seven days after challenge; the third survived to the end of the observation period. Single animals in both the sixth and tenth challenge control groups died untreated from acute trypanosomiasis, forty-five and thirty-four days respectively after challenge.

DISCUSSION

The virulence of the strain of *T. congolense* used was successfully maintained throughout the experiment, to judge by the uniform timing of its first appearance in the blood of the control cattle and the deaths within eighty-seven days after challenge of four of the five controls left untreated. The severity of infection by a syringe challenge of this type is recognised to be less than exposure to heavy natural or artificial fly infection by *Glossina* spp. Smith (1959) exposed eight control cattle to very heavy natural challenge; all showed positive blood films nine to thirteen days later and three of these animals, which were left untreated, died about fifteen days after infection was observed. Robson and Cawdery (1958), working in an area of high natural challenge, found the mean period between exposure of forty control cattle and the first appearances of trypanosomes to be 23·90 days. Seven of these cattle were allowed to die and the mean period between the first positive film and death was 68·5 days. The latter observation is similar to the results with control animals recorded in this paper; however, the protection afforded by 2 mg./kg. Prothidium Bromide against the challenge in Robson and Cawdery's experiment was very much shorter than in the present trial. The syringe challenge used probably gives a useful indication of the relative merits of the drugs under test in the face of light to moderate natural tsetse fly challenge.

A distinction must be drawn between the standard used when comparing the efficiency of prophylactic drugs and that employed as a basis for recommendations on the field use of any drug. In the latter case, it is most important that re-treatment should be carried out before persistent infections begin to occur. In the experiment described above, the first persistent infection in a group of treated animals has been regarded as the critical point so far as the prophylactic value of the drug at that particular dosage level is concerned. However, Robson and Cawdery (1958) calculated "the average number of days from exposure to the first positive slide of the first five animals in each group to 'breakthrough' " as an arbitrary means of comparison of the drugs used, while Smith (1959) compared the efficiency of prophylactic trypanocidal drugs by means of log-transformed data.

Under these conditions, Prothidium Bromide protected thirty-seven cattle against a varying number of challenges for 215 days and Antrycide Pro-salt protected thirty-five cattle for 258 days. This result agrees closely with that of Robson (1958) who exposed twenty cattle treated with 2 mg./kg. Prothidium Bromide to a light natural fly challenge. The first "breakthrough" occurred after 216 days exposure and two further animals had become infected by the time the experiment ended after 261 days. His results with Antrycide

Pro-salt are not comparable, as he gave bi-monthly treatments. Both Smith (1959), as well as Robson and Cawdery (1958), recorded shorter periods of protection with these drugs used at the same dosage levels. Out of eight cattle treated with Prothidium Bromide, Smith observed the first "breakthrough" after forty-seven days and all the cattle were positive after ninety-one days; the first "breakthrough" in sixteen cattle treated with Antrycide Pro-salt occurred after forty-three days and, with one exception which remained free to the end of the observation period, the rest were all positive after seventy-five days. Out of fourteen animals treated with Prothidium Bromide, Robson and Cawdery observed the first "breakthrough" after forty-seven days and only three cattle were still negative when the group was removed from challenge after 108 days. Their results with Antrycide Pro-salt are not comparable, since they gave bi-monthly treatments. These shorter protection periods might be attributable to the heavier challenge by tsetse fly or greater virulence of metacyclic trypanosomes. Although of shorter duration, the ratio of the protection periods conferred by Antrycide Pro-salt and Prothidium Bromide in the experiment of Smith is similar to that in the present experiment.

There appears to be no correlation between the number of challenges an animal receives and the duration of protection. It seems that the rate of elimination of the drugs by normal metabolic processes is the critical factor in determining the duration of protection.

The significance of transient infections is obscure. Of the five cattle in which this phenomenon was observed, the earliest persistent infection did not occur until 125 days later and all five received at least a further four challenges before regular appearance of parasites in the peripheral blood was established. It seems unlikely that the drug is not protecting completely and that suppressed infections may occur which can seldom be detected in the blood stream; if the drug failed to eliminate trypanosomes completely at the stage when a transient infection was noted, it seems improbable that a further three challenges could be sustained without even a transient parasitaemia being observed.

In discussing the occurrence of transient infections in an earlier experiment (Leach *et al.*, 1955), it was suggested that this phenomenon might be due to infection by mechanical transmission. Biting-flies are prevalent in Upper Nile Province during the rains (May to October) and act as vectors of bovine trypanosomiasis in the absence of *Glossina* spp. (Buxton, 1955). In the present experiment, four of the five transient infections observed occurred in the second half of the dry season, when biting-flies are relatively scarce, and the fact that the concentration of either drug under test was sufficient to require a further four syringe challenges before persistent infections were established appears to make this solution untenable.

Robson and Milne (1957) observed that treatment with drugs of the Prothidium series could lengthen the incubation period of *T. congolense;* nevertheless, the longest incubation period recorded by them was fifty days. Since the five cattle in the present experiment were challenged roughly every four weeks, their earliest persistent infection might well be attributable to the third syringe challenge after transient infection had been observed. This scarcely affects the argument against mechanical infection by flies. Smith (1959) also discusses this problem, although the periods he mentions during which blood films remained negative, following the observation of trypanosomes on a single occasion. were of considerably shorter duration than those recorded in this experiment. Assuming that organisms are constantly present in the peripheral circulation, he considers the chances of demonstrating trypanosomes in thick blood films are not high and recommends very frequent blood film examinations. We suggest that, if repeated examination of films fails to reveal the organisms, after they have been observed once, a substantial quantity of blood should be inoculated into a clean control animal. This should establish with reasonable certainty whether the parasites are present in the peripheral circulation.

If a waning concentration of a drug is insufficient to eliminate all the trypanosomes received in a particular challenge and the surviving organisms lack the virulence or numbers to establish a progressive, drug resistant infection, they might initiate an artificially induced state of premunity, on a parallel with the method of immunisation practiced with some other protozoal infections, using artificial inoculation of the parasite controlled by drug treatment. This hypothesis at present lacks experimental proof and, since the animals under observation were on a satisfactory nutritional plane, further moderate challenges might reasonably have been expected to reinforce such a premunity, rather than result in a persistent infection suggestive of the onset of a fatal attack.

While a completely satisfactory explanation of these transient infections is lacking, it seems that the limitations of the method of examination, as remarked on by Smith (1959), are of particular importance. There is, however, no evidence at present that the sporadic occurrence of transient infections is of major importance in judging the merits of prophylactic drugs. It follows that the present method of assessing prophylactic efficiency by observing the period between treatment and persistent infection by means of routine blood film examinations, having regard to the type of challenge and other conditions of each experiment, gives a satisfactory criterion of prophylaxis, unless it can be demonstrated that transient infections represent a danger as a source of incipient resistant strains.

Significant differences between the live weight gains of groups of apparently healthy cattle treated with various drugs and kept under identical conditions are probably a useful indication of the degree of sub-clinical toxicity resulting from administration of these compounds.

CONCLUSIONS

The prophylactic qualities of Antrycide Pro-salt and Prothidium Bromide against bovine trypanosomiasis were compared by subjecting groups of treated cattle to regular, repeated syringe challenge with *T. congolense.* The first persistent infection in a beast treated with Prothidium Bromide occurred 215 days after treatment and eight days after this particular group of cattle received their fourth challenge. The first persistent infection in an ox treated with Antrycide Pro-salt was observed 258 days after treatment and twenty-four days after this group received their seventh challenge.

Challenges at intervals of four weeks were continued until fifty-four weeks after treatment and details are given of all transient and persistent infections observed. The significance of the former is discussed.

Three of six cattle inoculated with 5 mg./kg. Prothidium Bromide died fifty-five to sixty-three days after treatment in circumstances suggestive of drug toxicity.

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SWAYBACK IN SOUTH-EAST SCOTLAND

I. Field Aspects

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INTRODUCTION

In 1956 one of us (R.M.B.) encountered a condition in lambs which resembled swayback clinically but did not show the cerebral demyelination which Innes and Shearer (1940) had concluded was the pathognomonic lesion of the disease (Barlow, 1956), and this prompted us to reinvestigate the epidemiology, pathology and biochemistry of this disease.

This work seemed particularly desirable in view of certain differences between the observations of previous British authors on swayback (Innes and Shearer, 1940; Shearer, Innes and McDougall, 1940; Shearer and McDougall, 1944; Allcroft, 1952; Allcroft and Lewis, 1957) and those of Australian workers on a similar disease known as enzootic ataxia, which like swayback can be prevented by administering copper to the pregnant ewe (Bull, Marston, Murnane and Lines, 1938; Bennetts and Beck, 1942; McDonald, 1942; Marston, 1952). For instance, the latter could find cerebral lesions in only a few cases and regarded demyelination in the spinal cord as being pathognomonic. Furthermore, enzootic ataxia has been regarded as the extreme manifestation of a simple dietary deficiency of copper whereas swayback has been classed as a conditioned copper deficiency disease since the figures obtained for the copper content of affected pastures were "normal" and generally much higher than those associated with enzootic ataxia. This difference is also reflected in the figures for the copper content of blood and liver. Our own observations, which have already been reported briefly elsewhere (Barlow, 1958a) agree more closely with those in Australia than with previous findings in Britain.

This paper describes our field investigations during the period 1956 to 1958 which included an analysis of the pastures and soil for trace elements. It was the first investigation of this kind to be carried out in Scotland. An account of our clinical, pathological and biochemical findings is given in the succeeding paper.

MATERIALS AND METHODS

During the winter of 1956-57 a circular letter was sent to all practising veterinary surgeons in the district served by the East of Scotland College of Agriculture asking them to report cases of ataxia in young or new-born lambs. The farms concerned were then visited and the flock history obtained. Affected lambs and their mothers were examined clinically and material obtained for pathological and biochemical examination. Fourteen of the 20 farms on which the disease had occurred were revisited and representative samples of the soil and pasture were taken for trace element analysis from areas where the ewes had been grazing during pregnancy. Samples were also taken from 3 control farms which were known to be free of the disease.

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Diagnosis of swayback. The following criteria were used for the diagnosis of swayback. (1) Ataxia in the newborn or young lamb. (2) Cavitation or gelatinous lesions of the cerebral white matter and/or a characteristic histological picture of chromatolysis and myelin degeneration in the brain stem and spinal cord. (3) Low copper status i.e. blood copper content of the affected lamb or its mother less than 60 *u*g./100 ml.; or liver copper content of the affected lamb less than 80 p.p.m. DW. (for lambs up to 9 weeks of age).

The clinical symptoms of the disease, although often highly characteristic, are not specific and a final diagnosis cannot be made until a pathological examination has been carried out. Other conditions which may be confused with swayback on clinical evidence alone include congenital absence of neurones, primary bacterial meningitis, clostridial enterotoxaemias, osteoporosis (Butler, Nisbet and Robertson, 1957a), hypomyelinogenesis congenita (Markson, Terlecki, Shand, Sellers and Woods, 1959), cerebellar atrophy (Innes, Rowlands and Parry, 1949), focal symmetrical encephalomalacia (Hartley, 1956; Barlow, 1958b) and spinal abscess. The last 3 conditions were encountered among the lambs examined in 1957, spinal abscess being the most common cause of locomotor disturbance apart from swayback.

Even when ataxia is associated with a low copper status a pathological examination is still necessary for diagnosis since this association is not confined to swayback (Butler, Nisbet and Robertson, 1957b).

Collection and analysis of soil and pasture samples. In general, the sampling technique followed was that recommended for hill pasture areas by Calder and Voss (1957) with the difference that 16 instead of 25 sub-samples were taken and pooled to form a composite sample from each area. An increase in the standard sampling error from one fifth to one quarter of the error associated with a single sub-sample is inherent in this modification, but this was considered to be justified in view of the fact that the areas sampled were all much more homogeneous as regards soil and vegetational type than the hill pastures for which the sampling scheme was designed.

Each area was divided into quarters and four samples were taken at random within each quarter. Herbage samples were taken by clipping and soil samples by auguring to a depth of six inches in the clipped areas, teams of two operators being used to avoid contamination of the herbage sample with soil. In the preparation of the herbage samples for analysis certain precautions were taken to avoid contamination with traces of metals. The samples were dried on aluminium trays at 80°C and milled in a Christy Norris hammer mill fitted with a stainless steel beater cross and screen.

Both soil and herbage samples were analysed spectrochemically for Pb, Mo, Co, Ni, Zn, V and Ti by the chemical concentration method and the variable internal standard arc technique described by Mitchell (1948) using a 0·5N acetic acid extract of the soil samples. Cu, Mn, Fe, Ba and Sr were also determined in the herbage samples by the method of Farmer (1950) which involves arc excitation of the plant ash without chemical concentration.

RESULTS

Incidence of Swayback

In 1957 swayback was diagnosed on 18 farms (Table 1) including Farm E from which ataxic lambs were obtained for examination in the previous year (Barlow, 1956). The incidence varied from about 1 to 50 per cent and in four instances was over 20 per cent. On ten farms the disease had not been known before and on the remainder the incidence had usually been much lower. Copper supplements in the form of mineral mixtures or licks containing from 200 to 1,800 p.p.m. copper were available to the ewes during pregnancy on six farms. The incidence of swayback on these farms varied from <1 per cent to 20 per cent.

An experiment was carried out during the winter of 1956-7 on Farm E to determine the effect of dosing the ewes with copper during pregnancy on the incidence of the disease. Two thirds of the ewes (about 100) were given 3 doses of 1 g. $CuSO_4.5H_2O$ in about 50 ml. of water by mouth at monthly intervals, the last dose being given about 10 days before lambing was due to begin. The incidence of swayback in their lambs was about 3 per cent while in those from the remainder of the ewes which formed the control group it was 23 per cent (Table 1). The incidence in 1957 appears to have been relatively high over the British Isles generally, for reasons which are not at present known. It was not maintained in the following year (or 1959) and the disease was diagnosed on only two additional farms (Q and R) making a total of 20.

Description of Farms

Details of the 20 farms on which swayback was diagnosed are summarised in Table 1. The disease occurred on both isolated farms and on groups of farms in loosely defined areas e.g. farms E, D and T are situated in the Lomond Hills, A, C and G in the Riccarton Hills and P, L, Q, H and J in the Pentland foothills. With one exception (Farm B) they were above the 500 ft. contour line with hill ground rising to between 1,000 and 1,300 ft. However, only two (K and O) could be classified as true hill farms, the remainder being of the marginal type with enclosed fields which had been subjected to varying degrees of cultivation. With two exceptions (C and F) they were situated along the south facing aspects of the main hill masses of the area and on thirteen farms the pastures grazed by the sheep had a predominantly southerly aspect.

The disease did not appear to be associated with any particular geological formation although where groups of farms were affected the geological characteristics were similar. Seven were situated on limestone formations and all four farms with an incidence of 20 per cent or over are in this group. Four farms were on sandstone, six on shales and clays and three on pyroxene andesite. However it should be borne in mind that this region of Scotland has been the site of much geological drifting from the N. West and the rock type quoted in Table 1 may not give a true indication of the soil type on a particular farm.

Stock and Pasture Management

On 17 farms the ewe flock consisted of Blackfaces and on 14 of them they were mated with Border Leicester tups to produce Greyface lambs. On two farms Greyface ewes were crossed with Suffolk tups and the other farm reared pure South Country Cheviot lambs. Where Blackface tups were used the flocks were maintained from home-bred lambs, but most of the other farmers bought replacements as ewe lambs or gimmers and cast them after the fourth or fifth lamb crop. Four farms carried "flying flocks," both ewes and lambs being sold in the autumn and fresh cast ewes bought in prior to tupping. In general lambing took place during the last week of March and the month of April.

There was no general policy for supplementing the diet during pregnancy. Some flocks were fed a concentrate ration with roots and hay towards the end of pregnancy as a routine, whereas others only received hay when weather conditions were exigent. Mineral supplements were available to the ewes during pregnancy on 8 farms and in 6 cases were rich in copper.

On marginal farms the sheep generally grazed upland areas of permanent pasture and had access to lower ground (rotational pastures and forage crops) in the winter months. On all these farms attempts had been made by liming and other measures to raise the productivity of the pastures. On 13 farms lime had been applied regularly at levels varying from 1½ to 10 tons per acre at intervals of 1 to 7 years. On 6 farms this had been combined with deep ploughing and reseeding with an occasional application of fertilisers such as basic slag and superphosphate. The soil pH values for the areas sampled are given in Tables 2 and 3. None of these is in the alkaline range.

Weather

Examination of meteorological data from the stations at Kirkcaldy and Peebles showed that the winters of 1955-56 and 1956-57 were generally mild with little frost and snow. Some severe weather did occur in the February of each year, but was followed by dry and sunny weather with rising temperatures producing conditions which were favourable for the early growth of pasture herbage. By contrast the winter of 1957-58 was very wet and cold throughout and these conditions persisted into lambing time when the growth of herbage was still considerably retarded.

TABLE 1

DETAILS OF SWAYBACK FARMS

Farm	*Type*	*Predominant aspect*	*Predominant type of rock*	*Pasture improvements*	*Breed of lambs*	*% Incidence of swayback*		*Cu supplement fed 1956–7*
						Average before 1957	*In 1957*	
A	Marginal	S.S.E.	Carboniferous limestone	Reseeded and limed	Blackface Greyface Other crosses	2 — 3	50	—
B	Marginal	N.W.	Carboniferous limestone	Limed 5 tons/acre every 5 yrs.	Greyface	< 1	20	+
C	Marginal/hill	N.E.	Carboniferous limestone Quartz-dolerite	Hill limed 1½ tons/acre in 1956	Greyface	0	2	—
D	Marginal/hill	S	Carboniferous limestone	Limed 5 tons/acre every 5 yrs. Heavy dressing with other fertilisers	Greyface	< 0·5	25	—
E	Marginal/hill	S	Carboniferous limestone Quartz-dolerite	Limed 10 tons/acre every 4 yrs.	Greyface Blackface	4 – 5	23	—
F	Marginal/arable	N.W.	Sandstone	Some reseeded. Limed 3 tons/acre in 1955	Greyface Blackface	0	2	—
G	Marginal	S	Clay	None	Greyface	< 1	2 – 3	—
H	Arable	S	Pyroxene andesite	Limed 5 tons/acre	Greyface	0	4	-
I	Marginal/hill	N	Clay Radiolarian chert	Not known (None for 3 yrs.)	Blackface	0	1	+

Farm	*Type*	*Predominant aspect*	*Predominant type of rock*	*Pasture improvements*	*Breed of lambs*	*% Incidence of swayback*		*Cu supplement fed 1956–7*
						Average before 1957	*In 1957*	
J	Marginal/arable	S	Old red sandstone	Limed 10 tons/acre in 1954 and 55	Greyface Halfbred	0	1·5	—
K	Hill	S	Old red sandstone	Limed 3 tons/acre in 1956	Blackface	0	1·5	+
L	Marginal/arable	None	Cornstone	Reseeded. Limed 3 tons/acre as necessary	Blackface	< 1	2	—
M	Hill	S	Clay and shales	None	Blackface Greyface	< 1	3	—
N	Arable	None	Clay	Fertilisers in rotation	Blackface Greyface	0	2	—
O	Hill	None	Old red sandstone	Reseeded. Limed 4 tons/acre in 1953 and 2 tons/acre in 1955	Cheviot	< 1	9	+
P	Marginal/arable	S	Pyroxene andesite	Limed 4 tons/acre every 7 yrs.	Greyface X Suffolk	0	13	—
Q	Marginal/arable	None	Pyroxene andesite	Not known	Blackface	<0·5	1	+
R*	Parkland	None	Clay and shales	Not known	Greyface	0	0	—
S	Hill	S	Shales	Reseeded. Limed 3 tons/acre in 1956	Greyface	< 1	6	+
T	Marginal	S	Carboniferous limestone Basalt	Reseeded. Limed 2 tons/acre. Other fertilisers used.	Greyface	0	7	—

*2% incidence of swayback in 1958.

Trace Element Content of Pasture

Some of the results for the 46 areas (generally fields) on the swayback farms and 11 areas which were sampled on control farms are given in Tables 2 and 3 respectively. Only the values for copper, molybdenum, lead and titanium are given here since those for the other elements which were estimated showed no features worthy of note.

The lead contents of some of the herbage samples were erroneously high owing to slight contamination of the samples with soil, abnormally high weights of precipitate obtained after treatment with x-hydroxyquinoline, tannic acid and thionilide being taken as evidence of contamination. These values were therefore omitted from the tables, but those for copper and molybdenum have been included since they are not usually considered to be affected appreciably by slight soil contamination, the reason being that, unlike lead, copper and molybdenum usually occur in the same order of concentration in soil and pasture dry matter (Mitchell, 1954). However, it is interesting to note that the high copper levels in Tables 2 and 3 are those for contaminated samples.

A much more sensitive index of soil contamination is provided by the amount of titanium in a herbage sample since the average concentration in the soil is about 10,000 times greater than in uncontaminated herbage. Thus it is obvious that even trace contamination of herbage with soil will affect the apparent titanium content. Statistical examination of the copper and titanium values for all the herbage samples reveals a highly significant correlation ($r=0{\cdot}715$, $P=<0{\cdot}01$) suggesting that these copper levels are in general higher than they would have been had it been possible to obtain samples completely free of soil. Since great care was taken to avoid obvious soil contamination when they were collected it is possible that this source of error also influenced the results reported by previous authors (Table 4) who do not appear to have given the problem such detailed consideration.

It is sometimes argued that the effects of soil contamination can be ignored since a contaminated pasture sample represents what the animal actually eats. However the argument ignores the probability that the availability of soil copper differs from that of the copper in the herbage and it also obscures the difficulties which arise when a comparison is made of the results for areas with different soils and climate such as Britain and Australia. Clearly it is only valid to compare results when they are not significantly affected by soil contamination.

If the samples which gave high precipitate weights are not taken into account there is no significant correlation between the titanium and copper values, and the remainder of the samples can therefore be regarded as being practically unaffected by soil contamination. Thus we can assess the copper status of the swayback pastures if we omit consideration of the apparent copper status of the samples marked "C" in Tables 2 and 3. When this is done, unfortunately, the number of samples in the control group (6) becomes too small for statistical comparison with the group from swayback farms and although the mean copper level is less for the swayback group, the difference is not significant.

The general level of copper content encountered in the swayback group is, however, of some interest. Of the 37 swayback herbages which were not regarded as being soil-contaminated, 23, i.e. about 62 per cent, had a copper content of less than 5 p.p.m. D.W., and the mean values for six out of the nine farms from which "uncontaminated" samples of herbage were taken, were at or below this level. These values are therefore similar to those for Australian pastures associated with signs of copper deficiency. The critical level for British breeds of sheep under Australian conditions and with a low molybdenum intake appears to be about 4 p.p.m. (Underwood, 1956) and on this basis some pasture on the majority of the swayback farms contained inadequate or borderline amounts of copper. Two of the swayback farms (E and M) had mean values of 4 p.p.m. or less.

There is an alternative method of assessing the copper status of the swayback pastures which takes account of both the contaminated and uncontaminated samples. If the regression of the copper content on the titanium content is calculated for all the pasture samples taken from swayback farms we can arrive at an estimate of the mean copper content of the 42 swayback pastures corrected for soil contamination. According to Mitchell (1954) the average Ti content of uncontaminated herbage is approximately 1 p.p.m. and if this value is substituted in the regression equation ($y=0{\cdot}0681x+3{\cdot}64$) the mean copper content corresponding to this content of Ti is 3·7 p.p.m.

All the Mo values in Table 2 appear to be "normal" and fall within the range 0·21 to 3·4 p.p.m. D.W. (mean = 1·25 p.p.m.). They are very similar to those previously published for swayback pastures (Table 4). While these values do not appear to be high enough to cause hypocupraemia there is some evidence that they could reduce copper storage in the presence of sufficient sulphate (Dick, 1953; Wynne and McClymont, 1955).

The results for lead were included in Tables 2 and 3 in view of previous interest in this element as a possible factor in the aetiology of swayback. The only unusually high herbage values obtained were for Farm M. These could not be attributed to soil contamination and would appear to represent the amounts actually present in the herbage. The lead content of the soil samples taken from this farm was not unusual and it is interesting to note that there was no general correlation between the lead values for herbage samples and those for the corresponding soil samples. It can be seen from Table 4 that many of the lead values recorded by previous authors are considerably higher than those obtained in cur investigation and this is

TABLE 2

pH AND TRACE ELEMENT CONTENT OF SOIL AND HERBAGE SAMPLES FROM SWAYBACK FARMS

Farm	*Date of sampling*	*Area no.*	*Soil*		*Herbage (p.p.m. D.W.)*			
			pH	*Pb (p.p.m. Air D.W.)*	*Cu*	*Ti*	*Mo*	*Pb**
A	29/5/57	1	5·5	1·1	8·9	85·9	1·20	C
		2	5·3	0·3	10·1	126	1·51	C
		3	5·4	0·4	25·5	275	2·34	C
		Mean	5·4	0·6	14·8	162·3	1·35	—
B	21/5/57	1	5·8	0·3	6·8	30·9	0·52	2·1
		2	5·7	0·4	7·3	75·5	0·76	C
		3	6·0	0·5	8·1	80·0	0·41	C
		4	5·0	0·4	8·7	72·1	1·20	C
		Mean	5·6	0·4	7·7	64·6	0·71	—
C	29/5/57	1	5·2	0·3	7·1	58·1	0·78	C
		2	5·2	0·7	8·7	117	0·79	C
		Mean	5·2	0·5	7·9	87·6	0·79	—
D	5/6/57	1	6·4	0·2	4·5	—	0·58	1·3
		2	6·3	0·6	3·1	46·8	1·01	6·2
		3	6·4	0·4	4·7	18·5	1·00	1·2
		4	6·3	0·7	14·8	35·0	2·20	C
		5	5·7	0·5	3·4	12·0	3·40	2·1
		6	6·4	0·4	4·5	14·2	1·98	1·8
		Mean	6·3	0·5	5·8	25·3	1·68	2·5
E	11/6/57	1	5·4	0·8	1·8	7·5	0·54	0·9
		2	5·7	0·3	5·1	21·5	1·86	C
		3	5·6	0·2	4·3	8·5	1·56	2·2
		4	5·6	0·3	4·8	39·0	0·93	1·2
		5	5·7	0·4	4·0	—	0·76	1·5
		Mean	5·6	0·4	4·0	19·1	1·13	1·5
F	28/6/57	1	6·0	0·4	3·19	8·5	0·21	0·9
		2	6·3	0·1	4·76	6·4	0·38	0·7
		3	5·7	0·4	4·89	8·4	0·78	1·1
		Mean	6·0	0·3	4·28	7·8	0·46	0·9
H	29/8/57	1	5·8	0·4	5·15	7·4	1·48	1·2
		2	5·5	0·2	4·35	7·2	0·35	1·8
		3	5·8	0·1	5·15	9·1	0·77	1·9
		4	5·3	0·2	5·38	25·9	0·41	2·4
		5	5·6	0·2	3·97	23·8	2·52	2·4
		Mean	5·6	0·2	4·80	14·7	1.11	1·9
I	29/8/57	1	6·8	0·7	5·56	29·3	1·85	1·2
		2	6·1	0·3	5·46	37·2	1·37	1·7
		3	6·3	0·2	4·86	11·4	1·98	0·5
		4	5·1	0·4	4·91	5·4	0·82	2·9
		5	6·2	0·3	6·93	15·2	2·05	0·9
		Mean	6·1	0·4	5·54	19·7	1·61	1·4
J	19/8/57	1	5·9	0·5	6·12	43·6	1·58	3·1
		2	6·0	0·8	4·10	42·7	1·36	4·0
		3	5·4	0·6	6·05	14·7	1·64	3·6
		4	5·3	0·3	3·78	—	2·31	5·5
		Mean	5·7	0·6	5·01	33·7	1·73	4·1

Table 2—*Continued.*

Farm	*Date of Sampling*	*Area No.*	*pH*	*Soil Pb (p.p.m. Air D.W.)*	*Herbage (p.p.m. D.W.)*			
					Cu	*Ti*	*Mo*	*Pb**
L	19/8/57	1	5·9	0·4	5·11	17·5	1·61	3·9
		2	6·0	0·2	6·51	10·9	1·26	4·0
		3	5·4	0·6	5·54	—	0·81	2·9
		4	5·3	0·3	7·05	22·1	1·39	2·2
		Mean	5·7	0·4	6·05	16·8	1·27	3·3
M	27/6/57	1	5·6	0·4	4·84	11·5	0·70	19·2
		2	5·9	0·4	3·59	10·9	1·36	34·3
		3	6·1	0·2	4·00	8·0	0·84	17·1
		4	6·0	0·3	2·88	6·1	1·22	12·4
		5	6·6	0·4	2·83	5·3	1·29	8·5
		Mean	6·0	0·3	3·63	8·4	1·08	18·3

*C indicates that the herbage sample was not suitable for lead analysis owing to soil contamination.

TABLE 3

pH AND TRACE ELEMENT CONTENT OF SOIL AND HERBAGE SAMPLES FROM CONTROL FARMS

Farm	*Date of sampling*	*Area no.*	*pH*	*Soil Pb (p.p.m. Air D.W.)*	*Herbage (p.p.m. D.W.)*			
					Cu	*Ti*	*Mo*	*Pb**
U	10/5/57	1	5·1	0·3	5·3	111	2·17	C
		2	5·8	0·3	3·5	110	1·34	C
		3	6·4	0·2	7·2	6·58	1·75	0·6
		4	6·2	0·2	5·5	90·0	1·27	C
		5	5·9	0·2	6·9	31·3	1·25	C
		Mean	5·9	0·2	5·7	82·2	1·56	—
V	6/6/57	1	6·5	1·0	4·8	35·0	0·23	0·1
		2	6·2	0·4	6·2	7·4	0.36	1·5
		3	6·7	0·4	4·4	9·0	0·57	0·9
		4	6·6	0·5	6·2	11·8	0·44	0·8
		Mean	6·5	0·6	5·4	15·8	0·40	0·8
W	11/6/57	1	5·3	0·4	4·6	7·1	0·53	8·8
		2	4·9	1·6	7·5	51·0	2·90	C
		Mean	5·1	1·0	6·1	29·1	1·72	—

*C indicates that the herbage sample was not suitable for lead analysis owing to soil contamination.

TABLE 4

COPPER, MOLYBDENUM AND LEAD CONTENT OF SWAYBACK PASTURES

Authors	*Time of sampling*	*No. of farms or samples*	*p.p.m. D.W.*					
			Copper		*Molybdenum*		*Lead*	
Innes and Shearer (1940)	Not given	8 farms	14–27	(19·5)	—		9–396	(104)
Shearer, *et al.* (1940)	Nov. Jan.	5 samples 5 samples	8–13 8–18	(9·8) (13·6)	— —		37–70 85–352	(50) (189)
Shearer and McDougall (1944)	April to June	21 samples	4.4–22·2	(14·1)	—		6–91·1	(70·4)
Hunter, Eden and Green (1945)	Not given	Not given	8– > 20	—	—		—	
Stewart, Farmer and Mitchell (1946)	August and December	10 samples 7 farms	4·4–9·2	(7·0)	0·34–1·2	(0·77)	—	
Mills (1951)	Not given	Not given	6–13		—		—	
Allcroft (1952)	Mostly April to June	7 farms	7–20	—	0·5–11	(4·1)	—	
Mills (1954)	Jan. and June	6 samples 1 farm	5·5–8·1	(7·0)	—		—	
Allcroft and Lewis (1957)	Mostly April to June	15 samples	8·4–20	(14)	0·46–3·6	(1·6)	—	
Barlow *et al.* (this paper)	May to August	46 samples 11 farms	1·8–25·5 3·63–14·8	(5·84) (6·32)	0·21–3·4 0·46–1·73	(1·25) (1·17)	0·46–34·3 0·88–18·3	(4·5)† (4·22)*

Mean values are given in brackets †Values for 36 samples *Values for 8 farms due to the fact that many of their samples were collected from lead mining areas in Derbyshire.

DISCUSSION

Except in abnormal years such as 1957 swayback does not constitute a serious economic problem in South East Scotland and this appears to be true for Scotland generally (Roberts, 1938; Wilson, 1959; Shanks, 1959). We could find no evidence to support the opinion quoted by Russell and Duncan (1956) that the incidence is particularly high on the limestone rocks of the Border counties. It is impossible to deduce a figure for the over-all incidence of the disease since many farmers are reluctant to report its occurrence, particularly if the incidence is less than about 5 per cent. However it should be emphasised that the disease is continually breaking out, sometimes in high incidence, on farms where it has been hitherto unknown, and since the aetiology of the disease has not yet been elucidated it is not possible to prevent these new outbreaks. Many farmers and veterinary surgeons have attributed the initial occurrence or an increased incidence of the disease to the improvement of marginal grassland by liming or by reseeding and the application of fertilisers and the information obtained in our survey supports this conclusion. Comparatively little is known of the effects of different systems of grassland management on animal health and this theory has not been tested experimentally, but it has been found that high intakes of calcium carbonate reduce the retention of copper by sheep (Dick, 1954). If this theory is true the extensive programme of grassland improvement which is being followed in Scotland may soon magnify the problem.

Dunlop and Wells (1938) concluded that a high incidence of the disease in a particular year is directly related to the abundance of pasture herbage during pregnancy. Our own observations are consistent with this hypothesis which would explain an association with grassland improvement and with the location of many affected farms on the south facing aspects of hill masses which was noted in our survey.

The factor (or factors) in pasture which have been supposed to cause the disease have not been identified. Although it was established that the incidence of the disease could be greatly reduced by increasing the dietary copper intake of the pregnant ewe, previous authors could find no difference between the copper content of swayback and healthy pastures which was usually above 5 p.p.m. and therefore apparently adequate for the sheep (Table 4). Our results are generally lower than those previously published particularly when the effect of soil contamination is eliminated and indicate that some pasture on the majority of the swayback farms contained inadequate or borderline amounts of copper. However it should be noted that our samples, like many of those examined in previous investigations, were collected in the spring and summer after swayback had been diagnosed. It is therefore difficult to assess the significance of the results in relation to the copper intake of the ewe during pregnancy and early lactation. This also applies of course to the values for molybdenum and sulphate. In this connection it is interesting to note that clover usually contains higher concentrations of copper than grass (Mitchell, Reith and Johnston, 1956) and in improved pasture during an open winter and spring an appreciable growth of grass could occur at a temperature below that at which clover can grow (i.e. <50°F.) thereby providing the ewe with a lower intake of copper than is indicated by the analytical data in Tables 2 and 3. It is obviously important that an attempt should be made to determine the copper, molybdenum and sulphur intake of ewes on swayback farms during pregnancy.

Since previous workers could find no abnormality in the copper content of swayback pastures they postulated that these contain some factor which depresses the normal utilisation of copper by the pregnant ewe, but there is at present no direct evidence that such a factor exists. Although Mills (1954) found that herbage samples from a swayback pasture contained less water-soluble copper than samples from normal pasture the significance of this finding is obscure since the samples were taken from moorland in Derbyshire and a ley in Kent, respectively, at different times of the year and so cannot be considered to be comparable in their botanical composition, state of maturity or soil environment. Each of these factors might profoundly affect the results obtained in investigations of this kind. When hay from swayback farms was fed to pregnant ewes at Weybridge swayback did occur in the lambs and this was interpreted as indicating that the causative factor was present in the hay (Green, 1951). However this conclusion may not be valid since the ewes themselves were taken from swayback flocks after tupping.

Thus a reappraisal of previous work and the results of our own investigation indicate that it might be worthwhile to reinvestigate the possibility that in some areas at least the low copper status associated with swayback is caused by a simple dietary deficiency of copper.

CONCLUSIONS

A survey of swayback in S.E. Scotland indicated that except in abnormal years such as 1957 the disease does not constitute a serious economic problem.

There was no evidence of a geological factor in the aetiology of swayback, but there did appear to be an association with the improvement of marginal grassland involving liming and/or reseeding so as to produce an earlier and more abundant growth of pasture herbage in the spring. Under certain circumstances this herbage might be expected to have a relatively low copper content.

Although it has been suggested that the annual incidence of the disease on an affected farm is directly related to the intake of pasture herbage during pregnancy there is at present practically no information on the intake during this period of copper or of elements such as molybdenum and sulphur which inhibit copper storage.

By comparing values for the copper and titanium content of herbage samples it was found that soil contamination which may not be evident to the naked eye can give erroneously high copper values.

The results for the copper content of representative pasture samples collected from farms on which swayback occurred suggest the possibility of a dietary deficiency of copper in the ewes, particularly when the effects of soil contamination have been eliminated, and in this way reveal a much closer similarity to the pastures associated with enzootic ataxia in Australia than has hitherto been apparent.

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SWAYBACK IN SOUTH-EAST SCOTLAND

II. Clinical, Pathological and Biochemical Aspects

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INTRODUCTION

The background to this investigation was discussed in Part I which was devoted to field aspects (Barlow, Purves, Butler and Macintyre, 1960). In this paper it was shown that the mean level of copper in swayback pastures, after applying a correction for the effect of soil contamination, was below the critical level suggested for sheep, and the suggestion was made that the low copper status associated with swayback in S.E. Scotland might be caused by a simple dietary deficiency of copper. This finding demonstrated a closer similarity to enzootic ataxia in Australia than had hitherto been apparent. It will now be shown that this similarity is also reflected in the clinical, pathological and biochemical data.

MATERIALS AND METHODS

A total of 79 lambs affected by swayback were examined over a 4 year period (1956 to 59). They were obtained from 25 farms widely distributed over S.E. Scotland. Eleven of the lambs came from outbreaks investigated in 1956, 52 from farms included in a survey which was carried out from 1957 to 1959 and 16 were sent to the veterinary investigation department of the Edinburgh School of Agriculture for routine diagnosis. Their age varied from 1 day to 6 months but most of them (70 out of 79) were less than 6 weeks old. The majority were Greyfaces; the remainder included Cheviots, Blackfaces, and Greyface Crosses with Suffolk or Border Leicester tups. For comparative purposes, histological studies were carried out on 6 apparently normal foetuses (25 to 50 cm. crown-rump length) and 13 healthy lambs from a few hours to 10 days old.

Blood samples were taken for copper estimation from some of the lambs, and also from their mothers and other ewes in the flock at the time of the first clinical examination. These were collected from the jugular vein with a specially made and cleaned all-steel needle into a copper-free Pyrex or polythene bottle containing purified ammonium citrate as an anticoagulant. Towards the end of the investigation a specially designed needle and bottle assembly was brought into use to facilitate the collection of the samples under field conditions and minimise contamination by adventitious traces of copper. It should be pointed out that the standard type of needle which has apparently been used by previous British workers is unsuitable for the collection of blood samples for copper analysis since it has a brass hub which contaminates the sample as it passes through (Marston and Lee, 1948; Butler and Newman, 1956; Beck, 1956).

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Part of this work was described in a Thesis submitted for the degree of D.V.M. & S., Edinburgh, 1959.

All organs and tissues were examined thoroughly post mortem. Most of the lambs were examined either soon after natural death or immediately after rapid decapitation through the atlanto-occipital space. It appeared unlikely that the latter would have survived any length of time under natural conditions. The brain, spinal cord and portions of the peripheral nerves and other tissues were removed and placed in fixative. Since it was found that this method of killing was responsible for the production of Marchi-positive artifacts a few of the lambs were killed by section of the vessels of the neck so as to leave the central nervous system (C.N.S.) intact. The axial skeleton with calvarium and vertebral arches removed to expose the C.N.S. was then suspended in fixative ("bulk fixation").

In the early stages of this work the C.N.S. and other tissues were cultured on ox-blood agar, glucose broth and meat-piece broth but since the results were generally negative this practice was discontinued unless bacterial infection was suspected.

Liver samples were taken from 47 lambs for copper estimation immediately after opening the abdominal cavity. The entire ventral (left) lobe of the liver was removed with stainless steel instruments and placed in a specially cleaned Pyrex dish which was then covered with a filter paper. Extreme care was taken to avoid contamination of the sample during collection.

Histological procedures. Transverse sections were cut through the superior frontal gyrus just anterior to the splenial sulcus, the parietal cerebrum just behind the level of the infundibulum, the occipital lobe, the midbrain at the level of the oculomotor nerve roots, the pons, the medulla at the level of the obex, and the spinal cord at the levels of first cervical and 10th thoracic segments, and from the cervical and lumbar enlargements. Longitudinal sections of the cerebellum were taken through the vermis and lateral cerebellar lobes and the lateral parts of the cervical and lumbar cord. Portions of the brachial plexus and sciatic nerves and stellate and other autonomic ganglia were also taken.

Longitudinal and transverse blocks were taken from the muscles of the back and limbs from as near standard sites as possible, in addition to representative blocks of liver, kidney, spleen and heart muscle. Formol-saline was employed for primary fixation, following which nervous tissue was transferred to a modified Bouin's fluid and other tissues to formol-corrosive. In a few instances formol-ammonium-bromide (F.A.B.) was used for the C.N.S. Tissues were dehydrated through an ethanol-benzene series and embedded in paraffin. Blocks for frozen sections were taken from sites adjacent to those of the paraffin blocks.

All material was examined using haematoxylin and eosin (H. & E.), supplementary techniques being employed as necessary. Myelin was stained using iron or lithium haematoxylins or copper phthallocyanin. Neurofibrils were impregnated by the methods of Holmes (1947) or Peters (1958) and Nissl substance was stained by the method of Spoerri (1948). Degenerating myelin was demonstrated by the Glees variant of the Swank-Davenport method (Glees, 1943) and fat by Sudan III or IV using either alcohol or triethylphosphate as a solvent. Glial fibres were stained by phosphotungstic acid-haematoxylin or Cajal's gold sublimate.

Estimation of copper. The samples of liver were prepared for analysis by cutting into pieces with stainless steel scissors and drying in an Edwards 3 PS Freeze Dryer and a stainless steel electric oven at 100 to 105°C. The tissue was then ground to a fine powder in a Hillman electric coffee mill which had a plastic container and stainless steel blade. About 50 g. were transferred to a sample bottle which was stored in a vacuum desiccator over calcium chloride until required for analysis.

Prior to the analysis, 2 ml. of whole blood or about 1 g. of the dry liver powder were wet ashed with a mixture of nitric, perchloric and sulphuric acids (Hopkin and Williams, Lead-free) on an electrically heated rack. Copper was then estimated spectrophotometrically with a dithizone method (Butler, 1952; Butler and Newman, 1956). This was preferred to procedures based on sodium diethyldithiocarbamate, on account of its greater sensitivity and freedom from interference by iron and other metals. It has not always been appreciated that the amount of iron which can be tolerated by the earlier diethyldithiocarbamate methods used in previous swayback investigations is limited, and, unknown to the analyst, this critical amount may be exceeded in certain samples which in consequence will appear to contain more copper than is actually present (Clare, Cunningham and Perrin, 1945). The errors arising from this source may be particularly serious in studies of copper deficiency, which causes the accumulation of abnormally large amounts of iron in the tissues. For instance, the concentration of iron in the liver of a copper deficient sheep may be several hundred times greater than the normal value (Marston, 1950). These errors may also be appreciable in the analysis of pasture samples which are contaminated by soil. Besides raising the iron content of the sample to very high levels soil contamination can also produce a significant increase in the copper content (Barlow *et al.*, 1960).

As was indicated above, strict precautions were taken to minimise contamination of the sample by adventitious traces of copper at all stages during the collection, storage, preparation and analysis. These were comparable with those adopted in Australia and were described in a previous publication together with the methods used for the purification of reagents (Butler and Newman, 1956).

RESULTS

Clinical Signs

The majority were live-born, full-term lambs, but a few were stillborn and in isolated cases there might have been actual abortion. It can be seen from Table 1 that about 40 per cent. were affected at birth and these were all severe clinical cases. In the remainder, clinical signs were not evident until they were several weeks old and in some cases appeared to be precipitated by docking and castration at the age of 4 to 6 weeks. In the more delayed cases viz. those over 6 weeks old, signs were often mild and only became apparent when the flock was being moved rapidly or excited. It appears that the older the lamb when clinical signs first appear the milder the course of the disease. Following the development of signs, growth rate was depressed and affected lambs developed a "tucked up", bedraggled appearance. The feet showed a tendency to uneven wear and overgrowth of horn. Whilst some lambs were able to survive, many died of secondary infections or starvation, or were destroyed. Survivors were usually unthrifty and stunted. They remained ataxic but appeared to gain some control over their disability.

The clinical appearance of the affected lambs was similar to that described for swayback by Stewart (1932) and Innes and Shearer (1940), and for enzootic ataxia by Bennetts and Beck (1942) and McDonald (1942). An attempt was made to carry out a more detailed neurological examination along the lines indicated by McGrath (1956) for the dog. The difficulties inherent in animal neurology are intensified in the case of sheep because they are unaccustomed to the proximity of the observer. In the more severe congenital cases there was often nystagmus, whilst the pupillary and corneal reflexes were sometimes depressed. Some lambs appeared blind and also deaf. Fine head tremors were noted in a few, together

TABLE I

SUMMARY OF RESULTS FOR SWAYBACK LAMBS

		Age when examined						*Total*	
		Under 3 wks.		*3 to 5 wks.*		*6 wks. to 6 months*			
		No.	*% of total*	*No.*	*% of total*	*No.*	*% of total*	*No.*	*% of total*
Congenital cases	severe	29	36·7	2	2·5	0	0	31	39·4
	mild	0	0	0	0	0	0	0	0
Post natal cases	severe	2	2·5	36	45·6	1	1·3	39	49·4
	mild	1	1·3	0	0	8	10·1	9	11·4
Cerebral lesions	macro	23	29·1	1	1·3	0	0	24	30·5
	micro	3	3·8	5	6·3	0	0	8	11·3
	none	6	7·6	31	39·3	10	12·7	47	59·5
Lesions in brain stem	+++	9	12·0	5	6·7	0	0	14	18·6
	++	15	20·0	25	33·3	2	2·7	42	56·0
	+	5	6·7	7	9·3	6	8·0	18	25·3
	none	0	0	0	0	1	1·3	1	1·3
Lesions in spinal cord	+++	19	34·0	7	12·5	0	0	26	46·4
	++	9	16·1	12	21·4	4	7·1	25	44·6
	+	0	0	0	0	5	8·9	5	8·9
	none	0	0	0	0	0	0	0	0
Copper in liver (p.p.m. D.W.)	Range	1·8— 8·0		1·8—11·3		7·0—106			
	Mean ± S.E.	5·6 ± 0·5		5·7 ± 1·0		26·3 ± 11·2			
	No. examined	18		12		9		39	
Copper in blood (μg./100 ml.)	Range	21·0 & 35·3		8·2—48·6		8·9—87·4			
	Mean ± S.E.	—		24·8 ± 6·0		39·6 ± 12·0			
	No. examined	2		4		6		12	

The following signs have been used to indicate the severity of lesions in the brain stem and spinal cord:

+++ Severe degeneration of myelin and/or chromatolytic nerve cells.
++ Moderately severe myelin degeneration and/or scattered chromatolysis.
+ Slight myelin degeneration and/or few degenerate cells.
— No lesions recognised.

with intermittent grinding of the teeth. The bleat and deglutition mechanisms were apparently not affected.

Completely paretic lambs were usually dull, with injected membranes and flaccid paralysis of the limbs, there being little or no reflex response to pressure on the interdigital fold. Less severely affected lambs were bright and alert with brisk pedal reflexes in the forelimbs. In the hind limbs the reflex response was variable though most commonly strong but delayed. Summation of subliminal interdigital stimuli was evident, and in the standing animal at rest considerable alteration in the position of a limb occurred before any reflex response was generated. When finally elicited, the response was strong and possibly exaggerated, thus leading to a new set of positional circumstances which in turn required correction. It was this phenomenon which appeared to be responsible for the ataxia. Neither spasm of muscle groups nor hypertonicity was observed, and thus these observations tend to agree more closely with those of McDonald (1942) for enzootic ataxia than with those of Innes and Shearer (1940), who attributed the ataxia of swayback to spastic paralysis.

There was no clinical evidence of anaemia or disease in other body systems and no evidence of specific disease in the mothers of affected lambs. At no time was wool dystrophy suspected but it should be borne in mind that changes similar to those described by Marston and Lee (1948) and Lee (1956) would be extremely difficult to detect in the Blackface fleece by visual inspection.

Macroscopic Lesions

Macroscopic lesions attributable to swayback were confined to the cerebrum and were present in only 24 (30·5 per cent) of the 79 lambs examined (Table 1). These had all been severe clinical cases, few surviving more than 14 days without hand feeding, and represented 71·9 per cent of the 32 cases under 3 weeks of age and 66 per cent of the 21 lambs dying in the first 48 hours of life without secondary bacterial infection. In many cases the external appearance of the brain was normal, though in some lambs up to 8 or 9 weeks of age the meninges showed varying degrees of non-specific oedema and vascular engorgement. In some instances the gyri were narrow and the brain appeared shrunken and failed to fill the cranium, but in others the cerebrum appeared swollen and tense, with broad flattened gyri.

The cut surface of the brains showed 4 types of abnormality which occurred both singly and in combination: (1) cavitation; 2) gelatinous lesions of white matter; (3) greying of the white matter with an indistinct demarcation from the grey substance; (4) dilatation of the lateral ventricles unassociated with stenosis of the foramina or cerebral aqueduct. These lesions were very similar to those described by Innes and Shearer (1940).

It should be emphasised that no macroscopic lesions of cerebral white matter were seen in 55 (69·5 per cent) of the 79 lambs examined. This group included 7 (33·3 per cent) of the 21 lambs which were most severely affected and all those cases which were more than 4 weeks old. No macroscopic lesions were ever observed in the brain stem or spinal cord.

Lesions in other tissues were confined to a few of the more severe congenital cases in which the frontal bones were thin and soft, another case in which the long bones were abnormally light and one in which there was unilateral muscular atrophy involving the left gluteal region.

Microscopic Lesions

Cerebrum. The histological appearance of cases showing gross lesions were of the following pattern. The cerebrum showed generalised congestion and oedema which was more marked in the younger animals. Capillary haemorrhages of a minor nature were occasionally seen. The pia-arachnoid was frequently distended with transudate and in a few cases there was slight proliferation of connective tissue cells. Changes in the cortex were mild and only present where the subjacent white matter was extensively involved. The organisation into layers and the differentiation into cell types was less well-defined than in the normal animal of the same age. The cells often lay in wide retraction spaces and had foamy vacuolated cytoplasm and large vesicular nuclei. Neuronophagia was not observed and satellite cells appeared to be fewer than in the corresponding areas in the normal animal (Fig. 1).

The larger cavities of the cerebral white matter were sometimes lined by a membrane composed of fibrous glia and nerve fibres (Fig. 5). This lining was not always present and was less well-defined at the periphery of the smaller cavities, or where such cavities bordered gelatinous areas. Frequently normal myelin extended right to the edge of a cavity and only rarely was Marchi-positive material demonstrated in the vicinity of such a lesion. Gliosis, as indicated by an increased frequency of paired cells, was seldom observed. These findings suggest that the membrane forms in the manner of a compression capsule rather than as the result of proliferative reaction.

The gelatinous areas consisted of a loose glial network with a very fluid matrix in which varying numbers of nerve fibres were present. Axis cylinders appeared to be more numerous than myelin sheaths. In appearance these lesions resembled gelatinous zones in the actively medullating foetal cerebrum (46 cm) except that in the former the axons were frequently coarser and sometimes appeared to be fragmented. The formed myelin in the peripheral areas of the gelatinous lesions in affected lambs and in the gelatinous areas of the actively medullating foetal cerebrum (Fig. 7) showed some affinity for Sudan dyes, and fine Marchi-positive and sudanophilic globules were present in the perivascular spaces and vascular endothelial cells. In general, the amount of Marchi-positive material observed in the cerebrum of swayback cases was no greater than in the normal lambs. Peripherally the gelatinous lesions faded into normal white matter through an ill-defined zone, which frequently appeared less cellular than normal owing to a diminished proportion of small darkly staining neuroglia. Such cells as were present had large vesicular nuclei and little cytoplasm and lay in wide retraction spaces. In this respect there was also more similarity to the foetus than to the normal lamb of the same age (Fig. 2). Gitter cells were present in these areas, but in number and distribution they were usually comparable with the normal animal.

Histological examinations revealed the presence of localised lesions of the gelatinous type in 10 per cent of the cases which macroscopically had appeared normal. It should be emphasised that no lesions of cerebral myelin were found in more than half the total number of ataxic lambs examined (Table 1).

Cerebellum. Unequivocal pathological changes were virtually absent from the cerebellum. The central core of white matter was often sudanophilic and contained numbers of Marchi-positive fibres and gitter cells. However their appearance did not differ appreciably from those in the normal animal.

Brain stem and spinal cord. Lesions of both nerve cells and fibres were observed constantly in the brain stem and spinal cord. The cell changes appeared to be confined to the large multipolar nerve cells of the brain stem and the ventral and lateral horns, and varied from mild chromatolysis, with swelling of the cell body and occasional vacuolation, to actual necrosis, the cytoplasm becoming structureless and of a "hyaline" appearance. In such cells the nucleus had often completely disappeared or remained as a pyknotic dot or shadowy outline at the margin of the cell. Intermediate stages showed margination of the nucleus with corrugation of its membrane. The Nissl substance of such cells formed a thin peripheral rim, and the neurofibrillary apparatus was also disrupted and appeared as short peripherally situated rods or as diffuse finely granular dust (Fig. 4). Whilst severely damaged cells were never observed in the normal animal, examination of the foetus indicated that large multipolar cells of this type go through a stage during development when they appear to be in the process of degeneration, showing changes somewhat similar to chromatolysis.

No particular prediliction site for cellular degeneration was apparent in the cases examined. Reference to a recently compiled atlas of the grey matter of the brain stem of the sheep (Palmer, 1958) has shown that severe chromatolysis and necrosis of nerve cells was most common in the red nucleus, vestibular nuclei and reticular formation. Less frequently the fifth motor, hypoglossal and pontine nuclei were involved. In the ventral horns of the cord, degenerate cells were most readily found in the lumbar enlargement. Whilst no attempt was made to count total numbers of frankly degenerate cells, they appeared to be most numerous in animals which had lived for 2 to 3 weeks. In older animals such cells appeared to undergo lysis or resolution since progressively fewer were seen. "Ghost" cells were occasionally seen in older animals but there was never evidence of neuronophagia.

Whilst degenerating cells of this type have been described in the spinal cord in swayback previously (Stewart, 1932), their occurrence has been given much more prominence in the Australian literature on enzootic ataxia (Bull, Marston, Murnane and Lines, 1938; Bennetts and Beck, 1942; McDonald, 1942).

Degenerating fibres as revealed by the Marchi technique were found scattered throughout the brain stem and also in the spinal cord. Rostrally their distribution tended to be diffuse, but progressive organisation into tracts occurred caudally. Aggregations of Marchi-positive fibres were commonly found in the medial longitudinal fasciculus, lateral lemniscus, restiform body, brachium pontis and trapezoid body. In the spinal cord they occupied chiefly the peripheral zones of the dorsal and ventral parts of the lateral funiculus and the sulcomarginal funiculus. They were also present, though less frequently, in the septomarginal funiculus (Fig. 6).

Examination of the spinal cords of normal animals by the standard procedure also revealed the presence of Marchi-positive material which frequently had a similar distribution. It differed, however, in that the globules were usually finer and more regular in size and did not show quite the same concentration and distribution as was evident in the swayback lamb (Fig. 3). The possibility that this material was artifact due to stretching of unfixed tissue (Smith, 1956) was considered. It was for this reason that the "bulk fixation" technique was introduced. Whilst this method did reduce the numbers of positive fibres in both swayback and normal material, those which remained in longitudinal sections of the cord in the normal lamb were often very similar to those in the affected areas of swayback cord (Fig. 3). Evidence that the Marchi-positive fibres in affected areas of swayback cord were in fact degenerating was found in the reduced density of myelin in appropriately stained sections, and the diffuse glial reaction which occurred in these sites in some older animals. In none of the cases examined were gitter cells considered to be more numerous than in the same areas of the normal animal of the same age. Marchi-positive fibres were also somewhat sudanophilic, but in the normal animal the sudanophilia decreased rapidly after birth. The positive Marchi reaction persisted for a longer period, a few stained fibres being present in the oldest normal lamb examined. King (1911) also noticed the presence of Marchi-positive fibres in the normal young lamb and this finding suggests that immature myelin may go through a stage when it gives a positive Marchi reaction.

Peripheral nerves. Degenerating fibres were rarely found in the peripheral nerves and no lesions whatsoever were observed in the dorsal root and sympathetic ganglia.

Viscera. Lesions in the viscera were confined to the kidneys of a few animals aged about 5 weeks. A small proportion of the more peripheral glomeruli showed atrophy of the tuft and proliferation and hypertrophy of the lining epithelium of Bowman's capsule, together with slight periglomerular fibrosis. This lesion appeared to be the same as that found in lambs suffering from a toxic osteoporosis (Butler, Nisbet and Robertson, 1957). No accumulations of haemosiderin were seen in the liver of any of the affected lambs.

Wasting was observed in the gluteal muscle of one lamb and had the histological appearance of disuse atrophy, the fibres being uniformly smaller in diameter than those of the unaffected side.

Bacteriological Findings

No specific bacteria were isolated except from one case with streptococcal meningo-encephalitis and a few with hepatic necrobacillosis.

Copper Content of Blood and Liver

The values obtained for the copper content of samples of whole blood and liver are summarised in Tables 1 and 2. In order to eliminate as far as possible any artificial elevation of copper levels, only values for lambs which, so far as could be ascertained, had not been hand fed or dosed are included in these tables. Results for ewes which had been dosed with copper are also omitted. One extremely high liver copper value of 540 p.p.m. D.W. was in fact obtained for a lamb which had been fed artificially, and the liver values for two other lambs in this group (17·3 and 121 p.p.m. D.W.) lie above the range of values obtained for the untreated lambs of the same age.

The lambs examined fall naturally into two groups according to their age, viz. under 6 weeks, and 10 weeks to 6 months, and it is interesting to note that the older lambs which were all mild postnatal or "delayed" cases had significantly higher levels of copper in the liver ($P < \cdot 01$) which is consistent with the normal variation with age reported by Cunningham (1946). Cases of this type may acquire normal levels from the diet after the onset of the disease. In Table 1, the values for the younger group have been subdivided at the age of 3 weeks and it can be seen that the liver values for these two groups are almost identical (mean values 5·6 and 5·7 p.p.m. D.W. respectively).

In Table 2, which summarises the blood values, a distinction is made between those lambs which were examined histologically and ataxic lambs from flocks in which swayback had been diagnosed but which were not themselves examined post mortem. The results for these two groups of lambs and their mothers are not, however, significantly different and are combined in Fig. 8 for comparison with the results obtained by previous British authors.

TABLE 2

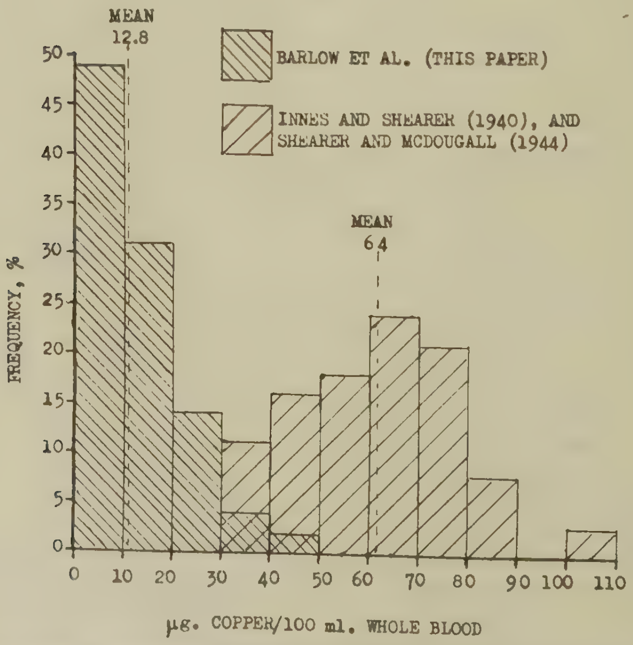
BLOOD COPPER VALUES OF SHEEP IN SWAYBACK FLOCKS

Results expressed in μg. Cu/100 ml. whole blood

Description	*No. examined*	*Range of values*	*Mean ± S.E.*
Mothers of swayback lambs	24	0·5—35·9	12·1 ± 1·8
Mothers of ataxic lambs*	25	0·5—41·4	13·5 ± 2·0
Swayback lambs (up to 6 wks. old) . .	6	8·2—48·6	25·9 ± 6·0
Ataxic lambs* (up to 6 wks. old) . .	1	21·8	—
Swayback lambs (10 wks.—6 months old)	6	8·9—87·4	39·6 ± 12·0
Ataxic lambs* (10 wks.—6 months old) . .	4	42·6—61·7	53·1 ± 5·0

*No post mortem examination was carried out on these lambs.

FIG. 8



Distribution of blood copper values obtained for mothers of swayback lambs.

Our observations suggest that liver copper values below 80 p.p.m. D.W. for lambs up to 6 weeks of age and blood copper values below 60 μg./100 ml. generally indicate a low copper status. Although these limits are tentative they are in agreement with the results obtained and the views expressed by previous authors (Cunningham, 1946; Marston, 1952; Harvey, 1952; Cuthbertson, 1954; Lee, 1956) and there is no doubt that the results obtained in the present investigation confirm the conclusion drawn by previous workers that the copper status of swayback lambs and their mothers is very low. It will be shown later that these values are much lower than those obtained by Shearer and his co-workers in their extensive investigations and are very similar to those associated with enzootic ataxia in Australia.

A detailed examination of the results summarised in Tables 1 and 2 showed no distinction between congenital and post-natal cases, and no correlation with the severity of clinical manifestations, or the severity and extent of lesions in the cerebrum, brain stem and spinal cord.

DISCUSSION

From the evidence presented in this paper there can be no doubt that the condition we investigated was the same as that described as swayback by Stewart (1932) and Innes and Shearer (1940), and as enzootic ataxia by Bull *et al.* (1938), Bennetts and Beck (1942) and McDonald (1942), but it can be seen that our findings resemble those of Stewart and the Australian workers much more closely than those of Innes and Shearer. The agreement between our observations and those of the Australian authors on clinical, pathological and biochemical aspects is indeed most striking and worthy of detailed discussion.

Innes and Shearer (1940) considered that the ataxia was due to a spastic paralysis, but McDonald (1942) found that this description could be applied to only one of the cases that he examined. In our investigation no hypertonicity and spasm of muscle groups was observed, and the essential defect appeared to be within the reflex mechanisms and to consist of a delayed response to stimulation of extero- and proprio-receptors. In some instances, the reflex response was exaggerated, but this may well have been the result of summation of repeated subliminal stimulation, as was shown to occur with the pedal reflex. It seems likely that it was this feature which led Innes and Shearer to apply the term "spastic paralysis" and Stewart to consider a primary proprioceptive defect as the cause.

Innes and Shearer (1940) concluded from their investigation that the lesions of swayback were "essentially those of cerebral demyelination with consequent destruction of the axis cylinders and later cavitation and secondary degeneration of the motor pathways". Although they commented upon the paucity of cellular reaction in the vicinity of the lesions they considered the myelin aplasia theory unacceptable as it did not explain "the porous areas, the gross cavitation, nor the myelin destruction demonstrated by positive staining reactions (Marchi and Scharlach R Methods)". It is evident in the light of our observations that their conclusions must be reconsidered.

The absence of demonstrable cerebral lesions in 59·5 per cent. of all our cases, and in 34 per cent of lambs dying in the first 48 hours of life without evidence of secondary bacterial involvement, strongly indicates that such lesions are not primary and indeed may have little clinical significance. This view is supported by the work of King (1911) who showed that the great motor pathways of the sheep originate at subcortical level, the cortico-spinal tract being rudimentary and apparently terminating in the first cervical segment. She also found that destruction of its cells of origin in the superior frontal gyrus had a negligible clinical effect.

In view of their presence in all our cases it is concluded that the lesions in the brain stem and spinal cord play a leading role in the causation of the ataxia. In the cases described in this paper, such changes were the most significant lesions observed and the only histological changes which could constitute a reliable basis for the diagnosis of swayback. Stewart (1932) and the Australian authors (Bull *et al.*, 1938; Bennetts and Beck, 1942; McDonald, 1942) constantly found similar lesions in the cord. Typically these lesions consist of a characteristic distribution of swollen Marchi-positive fibres which in some instances become frankly demyelinated together with degenerative changes in the large multipolar nerve cells of the brain stem, ventral and lateral horns. Innes and Shearer (1940) reported similar cell changes in the red nucleus, but they apparently did not observe them elsewhere. This might be explained by the age of the lambs examined. Frankly necrotic cells in our series of cases were usually found scattered amongst groups of apparently healthy cells and though no attempt was made to count the numbers they appeared to be more numerous in lambs 2 to 3 weeks of age than in the new-born animal. This suggests that they may result from retrograde, or possibly, trans-neuronal degeneration and may be secondary to lesions in the myelin sheaths. In this respect it is interesting to note that a few Marchi-positive fibres were still present in a case 6 months of age though in this instance the possibility of artifact cannot be ruled out.

If the anatomy of the sheep's spinal cord is similar to that of other mammals it would appear that the blackened fibres may be part of the rubro-, vestibulo-, and reticulo-spinal tracts, a view which is supported by the coincident involvement of their cells of origin. The first of these has been considered to be the great motor pathway of the sheep whilst the other two, in man, are concerned with the maintenance of muscle tone, and transmission of facilitating and inhibiting impulses respectively (Ranson, 1953). Lesions in these tracts could well account for ataxia as seen in swayback and would support the interpretation of the clinical signs already suggested.

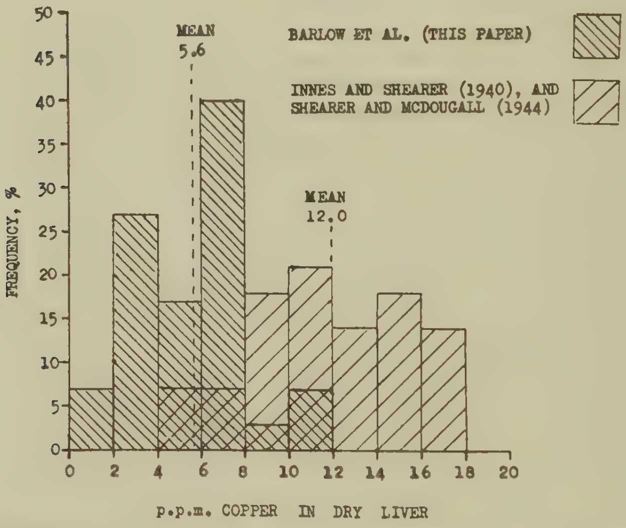
It seems unlikely that defects in the afferent pathways are of primary importance in swayback because of the relative infrequency and inconstancy of changes in the peripheral nerves, our failure to demonstrate lesions in the dorsal root ganglia and (in view of the findings in the normal animal) the equivocal nature and inconstancy of Marchi-positive fibres in the septomarginal funiculus. However, in the light of present knowledge of the internal anatomy of the sheep's nervous system such a view must be accepted with caution, since blackened fibres in the constantly involved lateral funiculus may belong to the spinocerebellar tracts transmitting proprioceptive impulses (Ranson, 1953).

Close consideration of the cerebral lesion suggests a theory of pathogenesis opposed to that of Innes and Shearer (1940). In our series: (a) the products of myelin degeneration in the form of fat-laden histiocytes were no more numerous in the overwhelming majority of cases than in normal young lambs; (b) apparently normal myelin extended up to the edge of well-formed cavities; (c) in general glia at the periphery showed no abnormally frequent evidence of division and (d) the glial fibres at the edge of the lesion had a laminated appearance more suggestive of a compression capsule than a zone of active proliferation. Markson, Terlecki, Shand, Sellars and Woods (1959) have stated that the products of myelin degeneration in the brain may be readily demonstrated by selective osmium methods, but these gave negative results for the cerebrum in this investigation.

The histological examination of the foetus and normal newborn lamb by identical methods indicated that there is a great deal of similarity between cases of swayback with the gelatinous cerebral lesion and normal lambs at an earlier stage of development. The staining reactions of myelin at subcortical levels also tend to support such a view. This suggests that the biochemical lesion which precedes the primary histological lesion inhibits the normal development of nerve cells, myelin sheaths and glia, especially during the most active growth phase of the foetal nervous system. With continuation of growth and the onset of function it is possible that these immature elements may degenerate and development be delayed or completely arrested in phylogenetically younger divisions of the C.N.S. This would account for the formation of the gelatinous and cavitated areas in the cerebrum since the period of medullation in this division of the brain coincides with that of its most rapid growth. This interpretation would also explain the delayed onset of signs in a proportion of cases. Thus it is suggested that the lesions of swayback are due to a neurodysgenesis which may involve nerve cells and glia as well as myelin sheaths although degeneration of the latter may also occur. This view is therefore opposed to that of Innes and Shearer who concluded that swayback is a demyelinating disease.

It can be seen from Fig. 8 that our results for the copper content of the blood of the mothers of swayback lambs are considerably lower than those obtained by Shearer and his co-workers at Cambridge (Innes and Shearer, 1940; Shearer and McDougall, 1944). Whereas 94 per cent of the blood copper values obtained in the present investigation fall below 30 μg./100 ml. (over-all mean = 12·8 μg./100 ml.) all those published by these authors lie above this level (mean = 64 μg./100 ml.) and over half of them (56 per cent) would be regarded as normal by our standards. The values obtained by Eden, Hunter and Green (1945) (range < 10 to 80 μg./100 ml., mean 30 μg./100 ml.) occupy an intermediate position between the two sets of results.

FIG. 9



Distribution of liver copper values of swayback lambs (congenital cases).

A wide difference is also shown by the blood and liver copper values for affected lambs. The mean of our liver values for lambs under 6 weeks of age is 5·6 p.p.m. D.W. and 91 per cent are less than 8 p.p.m. D.W. while the mean (12·0 p.p.m. D.W.) and 85 per cent of the values obtained by Shearer and his co-workers lie above this level (Fig. 9). (The latter do not state the age of the lambs they examined, but from the text it appears that the majority were acute cases and therefore were probably under 6 weeks of age.) Values ranging from 3·2 to 8·9 p.p.m. D.W. about a mean of 6·3 were recently recorded for 11 swayback lambs of this age group by Allcroft, Clegg and Uvarov (1959) and it can be seen that they are in very good agreement with our results. It is of course impossible to determine whether this wide difference between our results and those obtained by Shearer and his colleagues represents a real difference between the animals examined or whether it is due to differences in the techniques used for the collection and analysis of the samples, but it should be pointed out that the technical improvements which were introduced in our investigation would be expected to produce lower and more accurate results.

Our results for blood and liver, like those for pasture samples (Barlow *et al.* 1960), are very similar to those associated with enzootic ataxia in Australia. For instance, Bennetts and Beck (1942) analysed the blood of 9 ewes with affected lambs and found that 7 of them had values below 10 μg./100 ml. The highest value obtained was 40 μg./100 ml. Marston (1952) summarised the findings of his group by stating that signs of copper deficiency appear when the blood copper level has fallen below about 30 μg./100 ml. Bennetts and Beck also recorded liver copper values for 5 affected lambs under 6 weeks old which ranged from 4 to 8 p.p.m. D.W. about a mean value of 6·4. Bull *et al.* (1938) obtained similar results for 6 affected lambs (range 2·6 to 9·8 p.p.m. D.W. mean 6·0) but did not state their age.

The exact role of the low copper status in the aetiology of swayback remains obscure. While there has been ample confirmation of the efficiency of copper supplements, either by mouth or by injection, in greatly reducing the incidence of the disease, there is as yet no direct experimental evidence that a dietary deficiency of copper, per se, will produce lesions in the central nervous system in any animal. The copper deficiency syndrome has now been reproduced experimentally with low copper diets on several occasions in non-ruminant animals e.g. rats (Frick and Lampl, 1953; Gallagher, 1957), puppies (Baxter and van Wyk, 1953; Baxter, van Wyk and Follis, 1953) and pigs (Follis, Bush, Cartwright and Wintrobe, 1955), but no nervous symptoms or lesions have ever been reported. Furthermore, in lambs affected by swayback there is no correlation between the copper status and the severity of the disease, and our own observations have confirmed those of Allcroft and Lewis (1957) that a low copper status can occur in flocks which are completely free from the disease.

CONCLUSIONS

A detailed examination of 79 lambs affected with swayback which were obtained from farms in S.E. Scotland showed that the pathognomonic lesions are microscopic and occur in the brain stem and spinal cord and not in the cerebrum as had been believed hitherto, thereby demonstrating a close similarity to enzootic ataxia in Australia. There were no cerebral lesions in 59·5 per cent of these lambs including 34 per cent of those which were most severely affected. Macroscopic lesions were seen in only 30·5 per cent of all cases examined and all these were congenital cases.

Comparative studies on foetal and normal newborn lambs suggested that the primary lesions cause an inhibition in the development of nerve cells, myelin sheaths and glia i.e. the condition is a neurodysgenesis rather than a demyelination as was suggested by Innes and Shearer.

Clinical examinations indicated that the ataxia may be due to delayed responses to extero-, and proprio-receptive impulses rather than to spastic paralysis which has been previously thought to be the cause of the ataxia.

The analysis of samples of blood and liver with improved techniques revealed a very low copper status in affected lambs and their mothers, and gave results which are very similar to those associated with enzootic ataxia, but which are considerably lower than those previously obtained in Britain by Shearer and his co-workers.

There was no correlation between the copper status and the severity of clinical manifestations or nervous lesions after the disease had appeared.

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LEGENDS TO ILLUSTRATIONS

Fig 1. (a) 1026/57. A portion of the cerebral cortex adjacent to a gelatinous lesion. This tissue is oedematous and there is comparatively little differentiation of the various cell types. (H. & E. × 260).

(b) 357/58. A portion of the cortex of a 46 cm. (crown-rump length) foetus. There appears to be a greater degree of cellular differentiation than is evident in Fig. 1(a). (H. & E. × 260).

(c) 2390/57. Portion of the cortex of a normal lamb 18 hours old. The tissue shows a high degree of cellular differentiation. (H. & E. × 145).

Fig. 2. (a) 1026/57. Subcortex of a swayback lamb showing a gelatinous lesion. There are fewer cells with small darkly staining nuclei than in 1(b). (H. & E. × 110).

(b) 357/58. Subcortex of 46 cm. (crown-rump length) foetus. Small darkly staining nuclei are numerous. The "oedematous" nature of the tissue is evident. (H. & E. × 110).

(c) 2390/57. Subcortex of normal lamb 18 hours old. Astrocytes are discernable, microglia are numerous and "paired" cells are not uncommon. (H. & E. × 260).

Fig. 3. (a) 791/58. Longitudinal section of the lateral funiculus of the spinal cord of a severe case. Tissue obtained by bulk fixation. In many instances Marchi-positive fibres are not so numerous. (Glees × 55).

(b) 611/58. Longitudinal section of lateral funiculus of the spinal cord of a normal lamb. Tissue obtained by the routine methods to show stretch artifact. The group of Marchi-positive fibres just beneath the meninges probably indicates the position of a nerve root. (Glees × 55).

(c) 267/58. Longitudinal section of the lateral funiculus of the spinal cord of a normal lamb. Tissue obtained by "bulk fixation". Note the reduction in the numbers of Marchi-positive fibres. Those that remain are often indistinguishable from those occurring in swayback. (Glees × 55).

Fig. 4. (a) 1026/57. Cell in the thalamus showing severe chromatolysis. The affected cell is grossly swollen and the cytoplasm has a hyaline appearance. A nuclear remnant is just visible at the periphery of the cell. (H. & E. × 260).

(b) 1110/57. Chromatolysis of a multipolar nerve cell in the thalamus. The adjacent cell is apparently normal. (Spoerri × 260).

(c) 1110/57. Chromatolysis of multipolar nerve cell in the thalamus showing dust-like degeneration of the neurofibrillary apparatus. (Holmes × 260).

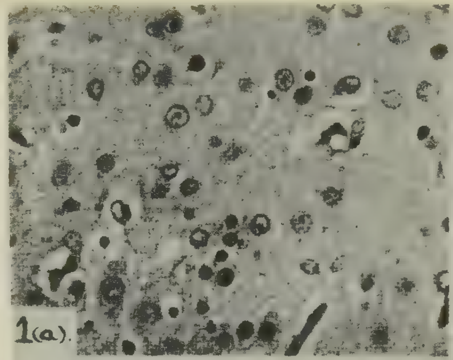
Fig. 5. (a) 1058/57. Periphery of a cavity in the subcortex. The glial fibres run parallel to the surface giving a laminated appearance suggestive of a compression capsule. This was the type most commonly seen. (Phosphotungstic acid haematoxylin × 145).

(b) 791/58. Proliferating network of fibrous glia at the periphery of a subcortical cavity. Such an active repair process was uncommon. (Frozen section, Cajal × 145).

Fig. 6. 795/58. Transverse section of the thoracic cord of a severe case showing the very typical distribution of Marchi-positive fibres. "Bulk fixation". (Frozen section Glees × 6).

Fig. 7. (a) 1026/57. Edge of a gelatinous lesion showing a gradual increase in the number of medullated fibres towards the periphery. A dilated perivascular space is present near the lower left-hand corner. (Frozen section Spielmeyer × 145).

(b) 357/58. Subcortex of 46 cm. foetus. Fine myelin sheaths and globules may be seen. There is a gradually decreasing density of stained fibres towards the cortex. (Frozen section Spielmeyer × 90).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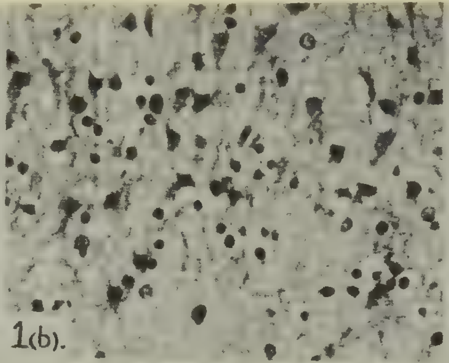
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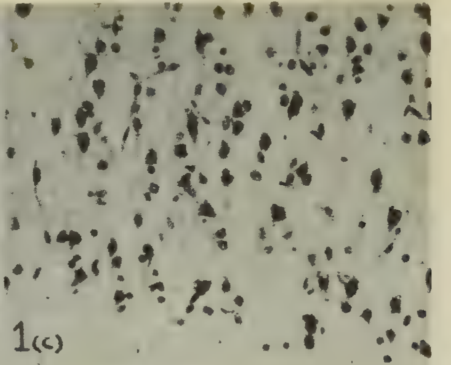
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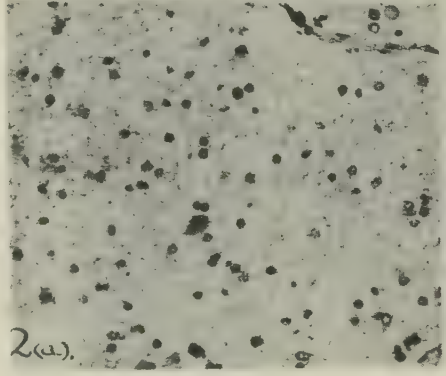
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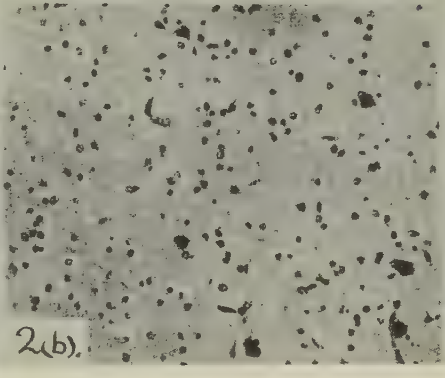
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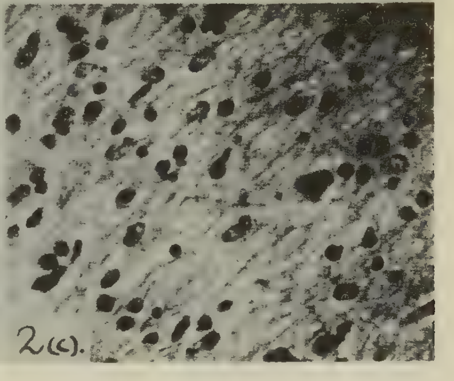
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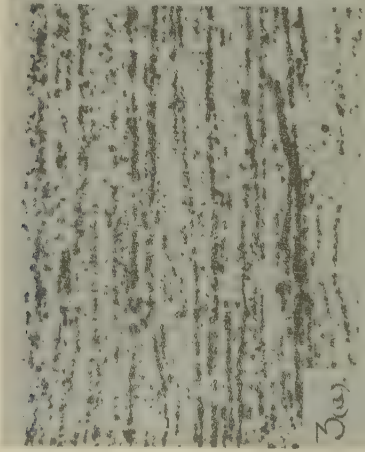
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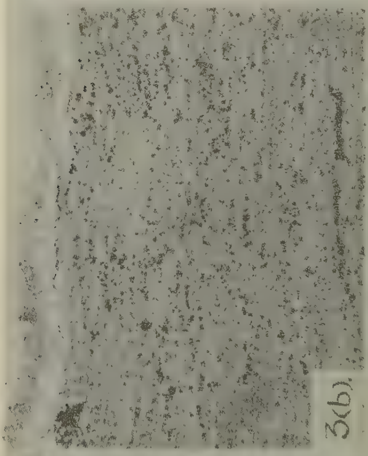
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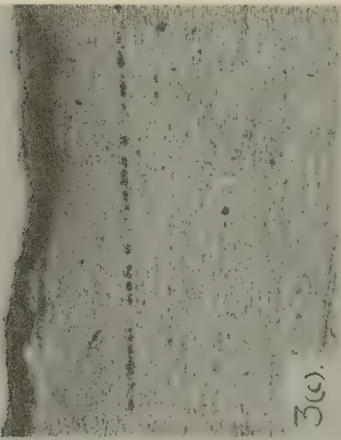
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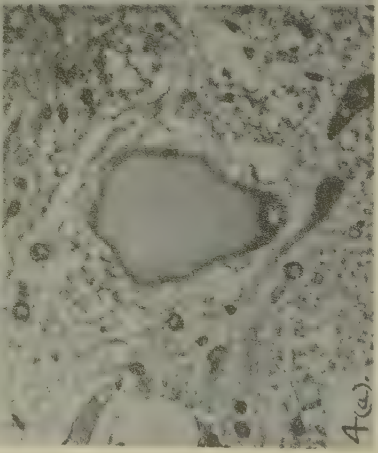
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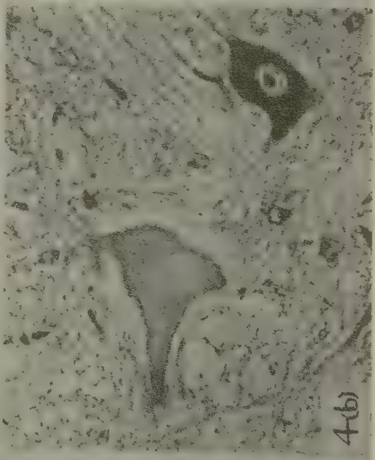
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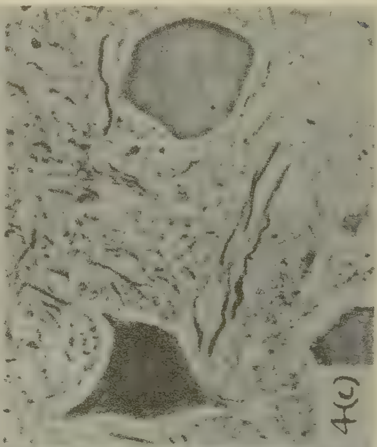
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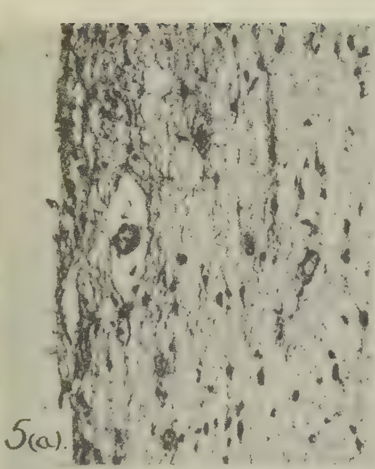
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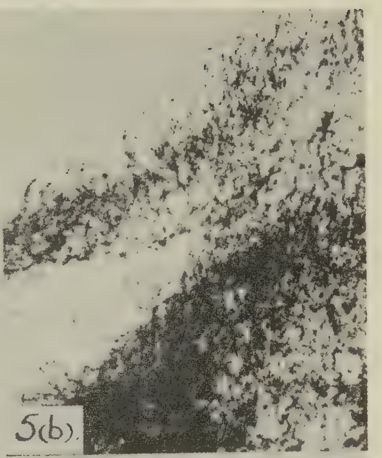
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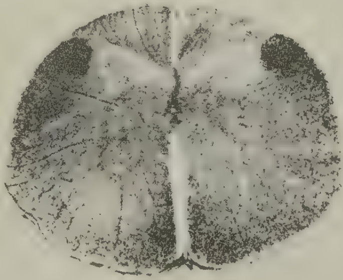
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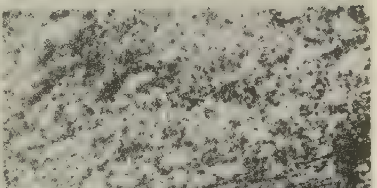
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VIRULENCE AND TOXICITY OF *PASTEURELLA HAEMOLYTICA* IN THE EXPERIMENTAL PRODUCTION OF OVINE SEPTICAEMIA

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INTRODUCTION

Organisms similar to those described as *B. bovisepticus* Group 1 by Jones (1921) may frequently be isolated from cases of disease in sheep and cattle and are now generally referred to by the name *Past. haemolytica.*

The literature contains observations by several authors on the production of disease and death in large domestic animals by intravenous injection of cultures of these organisms. Tweed and Edington (1930) found that an intravenous dose of about $50{,}000 \times 10^6$ organisms produced death in a calf 36 hours after inoculation. A prominent post-mortem finding was the presence of numerous small, subcutaneous haemorrhages. This experiment was repeated with similar results. Dungal (1931) produced profuse sweating and shivering in a pony by injecting intravenously 10 ml. of 12-hour broth culture. The animal had apparently recovered after 3 hours. Florent and Godbille (1950) reported the death of a sheep following the intravenous injection of a large dose. Stamp, Watt and Thomlinson (1955) described a *Past. haemolytica* septicaemia of lambs occurring most frequently in late summer. The disease was almost exactly reproduced in 7 to 9-month-old lambs by the inoculation of diseased tissue suspensions, primary surface culture suspensions and broth cultures. Surface culture suspensions contained an average of 1500×10^6 organisms per ml. and were used in doses of 1 to 6 ml. Broth cultures containing $10^{8 \cdot 5}$ organisms per ml. were injected in doses of 3 to 12 ml. Death and the typical lesions usually resulted within 12 hours. Occasionally a severe polyarthritis occurred instead or the inoculation was without observable effect. Smith (1957) pointed out that the septicaemia of lambs constituted the type of disease which might be expected to result from toxic concentrations of a Gram-negative organism and that the large doses which had been used in reproducing the condition by intravenous inoculation might not be far removed from the toxic level in terms of bacterial generations. Biberstein and Kennedy (1959) reproduced the septicaemic disease of lambs by the intravenous injection of doses ranging from 500×10^6 to 2000×10^6 organisms. They stated that such doses, although large, were essential for setting up the experimental disease. Smith (1959) described 2 types of *Past. haemolytica,* designated A and T. The majority of cases of enzootic pneumonia yielded Type A organisms in pure culture while all cases examined of the septicaemic disease of older lambs as described by Stamp *et al.* (1955) were associated with Type T infections. Smith (1960) reported cases of generalised Type A infection in lambs within the first few weeks of life and demonstrated the high susceptibility of animals in the same age group and the high resistance of adult sheep to both intraperitoneal and intravenous inoculation of a freshly isolated Type A strain.

This paper records experiments on *Past. haemolytica* designed to investigate further its pathogenicity in relation to the septicaemic infections of older lambs described by Stamp *et al.* (1955). Information is presented on the virulence by intravenous inoculation of a freshly isolated Type T strain in 7-month-old lambs and adult sheep. The implications of using large doses are discussed in the light of an experiment on toxicity of the organism.

MATERIALS AND METHODS

Experimental animals. Lambs between 6 and 7 months of age were bought for the experiments and used within the period of one month. Adults consisted of 18-month-old sheep from the Moredun Institute flock. All animals showed normal temperatures prior to inoculation.

Strain of Past. haemolytica. The strain used for inoculation was a Type T organism isolated from a 6-month-old lamb which died following a natural septicaemic infection. The bacteriology of this case is described under Results where the animal is referred to as Lamb 3. The organism was preserved at −50°C in pieces of infected lung tissue until required for use several weeks later.

Inocula. These consisted of dilutions of 5-hour infusion broth cultures. Immediately before injection counts of viable bacteria in inocula were performed by the method of Miles and Misra (1938). The average of 5 replicate counts was taken to be the true value. Killed culture was prepared by heating at 55°C for 30 minutes in a water bath. This was carried out immediately before inoculation and a large sample of the heat-treated material was cultured to confirm that sterilisation was complete. Dilutions of living and heat-killed culture for inoculation were made up, using broth as the diluent. All inocula were administered by the intravenous route.

Blood cultures. 5 ml. volumes of blood were withdrawn aseptically from the jugular vein and inoculated into 50 ml. volumes of broth. After incubation these cultures were examined for the presence of *Past. haemolytica.*

Counts of viable bacteria in tissues. The method used was that described previously by Smith (1960).

RESULTS

Bacterial Examination of Field Cases

Three 6-month-old lambs which died from natural septicaemic infections of *Past. haemolytica,* Type T, were examined with a view to finding the degree of bacterial invasion of certain organs. Autopsies were carried out immediately after death and counts of viable organisms in lung, liver, spleen and kidney performed. The symptoms and lesions were similar to those described by Stamp *et al.* (1955). Lamb 1, which died in 1957, came from a Berwickshire flock of 200 six-month-old lambs in which 12 deaths from the disease occurred during a period of 5 days. Lambs 2 and 3 belonged to a group of 250 six-month-old lambs which were bought by the Moredun Institute in the autumn of 1959. Six deaths from Type T infection occurred shortly after purchase. The strain isolated from Lamb 3 was subsequently used for the experiments on virulence and toxicity which are described later. Table 1 shows the numbers of viable bacteria present in the various tissues examined. The counts in lung, liver and spleen were extremely high while those of kidney tissue were very much lower.

TABLE 1

DISTRIBUTION OF BACTERIA AT DEATH IN 3 NATURAL CASES OF *PAST. HAEMOLYTICA* SEPTICAEMIA

Lamb No.	*Counts of viable bacteria in tissues (millions per g.)*			
	Lung	*Liver*	*Spleen*	*Kidney*
Lamb 1	450	550	65	0·1
Lamb 2	1,150	900	450	N
Lamb 3	14,500	275	300	0·03

N = Not done.

Intravenous Inoculation of Lambs and Adult Sheep with Graded Doses of Past. haemolytica

Details of the strain of *Past. haemolytica* used have already been given. Five groups of 4 lambs were inoculated intravenously with doses of $8{,}200 \times 10^6$, 82×10^6, 82×10^4, 82×10^2 and 82 viable organisms respectively. The dose volume in each case was 10 ml. and the largest dose consisted of 10 ml. of undiluted 5-hour infusion broth culture. Similar groups of adults were treated in the same way except that the smallest dose was omitted. The results are given in Table 2. All the lambs and adult sheep which received the largest dose died but none of the smaller doses proved fatal. Of the animals which had received lethal doses several showed accelerated respirations when examined 2 hours after inoculation. By the 5th hour one lamb was dead, two were prostrate and all animals destined to die showed respiratory distress, elevated temperatures and mild to severe diarrhoea. At the 18th hour the last of the 4 fatally infected lambs died and the 4 adults which received the same dose level succumbed between the 19th and 30th hours. Apart from a somewhat longer survival time in adults than in lambs, the experiment did not demonstrate in terms of death and survival any difference in resistance associated with age. However it will be seen from Table 2 that when blood cultures, made 24 hours after inoculation, were examined a clear difference emerged. Circulating organisms were present in the majority of surviving lambs, but in only one adult. At this time about half the lambs showed temperatures in the region of 105°F but none exhibited visible symptoms. The only adult to show an elevated temperature was the one whose blood culture proved to be positive. On the 4th day blood cultures from only 3 lambs were still positive and on the 7th day the blood of all experimental animals appeared to be free from bacteria.

The post-mortem appearances of the dead animals were similar to those described by Stamp *et al.* (1955). Counts of viable organisms in the tissues were performed immediately or shortly after death and the results are presented in Table 3. The counts obtained from liver and spleen tissue were in a number of instances considerably smaller than those found in natural cases of the disease (compare Table 1).

TABLE 2

INTRAVENOUS VIRULENCE TITRATIONS IN LAMBS AND ADULT SHEEP

Dose of viable organisms	*Lambs*		*Adults*	
	Deaths	*+ve blood cultures after 24 hrs.*	*Deaths*	*+ve blood cultures after 24 hrs.*
8,200 × 10^6	4/4	....	4/4	....
82 × 10^6	0/4	3/4	0/4	1/4
82 × 10^4	0/4	3/4	0/4	0/4
82 × 10^2	0/4	3/4	0/4	0/4
82	0/4	0/4	N	....

N = Not done. Dose volume = 10 ml.

TABLE 3

DISTRIBUTION OF BACTERIA AT DEATH FOLLOWING INTRAVENOUS INOCULATION OF LARGE DOSES

No. of animals	*Counts of viable bacteria in tissues (millions per g.)*		
	Lung	*Liver*	*Spleen*
4 lambs	80	55	2
	47	2	10
	475	12	50
	300	50	17
4 adult sheep	850	110	125
	165	4	120
	325	775	72
	275	8	22,750

Each animal received 8,200 × 10^6 viable organisms in a dose volume of 10 ml. (see Table 2).

TABLE 4

LETHAL EFFECT OF LIVING AND HEAT-KILLED CULTURE INOCULATED INTRAVENOUSLY INTO LAMBS

Dilution of 5-hour broth (Dose volume = 100 ml.)	*Living culture*		*Heat-killed culture*	
	Deaths	*Period between inoculation and death (hours)*	*Deaths*	*Period between inoculation and death (hours)*
1/1	N	....	2/2	5, 6
1/2	N	....	2/2	5, 10
1/10	2/2	8, 10	1/2	7
1/100	2/2	17, 68	0/2	....
1/1000	0/2	....	N	....

N = Not done.

Undiluted broth culture had opacity of less than Brown's No. 1 and unless sterilised by heat contained 410 × 10^6 viable organisms per ml.

Intravenous Inoculation of Lambs with Graded Doses of Living and Heat-Killed Past. haemolytica

A 5-hour infusion broth culture of the experimental strain containing 410 × 10^6 viable organisms per ml. and having an opacity of less than Brown's No. 1 was titrated intravenously both in the living state and after sterilisation by heat at 55°C for 30 minutes. Three pairs of lambs received doses of 10 ml., 1 ml. and 0·1 ml. of living culture, suitably diluted to make dose volumes of 100 ml. per animal. Four pairs of lambs received doses of 100 ml., 50 ml., 10 ml. and 1 ml. of heat-killed culture and again each dose volume was made up to 100 ml. The results are shown in Table 4 and clearly demonstrate that the lethal dose of killed culture was only a low multiple of the lethal dose of living culture. Thirty minutes after inoculation the animals appeared normal, but 4 hours later a number of them exhibited marked symptoms which included respiratory distress, diarrhoea, elevated temperature and prostration. These symptoms were shown by lambs which received heat-killed culture as well as by those infected with living organisms. The majority of deaths occurred within 12 hours. As in the previous experiment 10 ml. of living culture constituted a fatal dose while 0·1 ml. did not. The latter dose did however produce positive blood cultures in each of 2 lambs for 6 and 8 days respectively after inoculation; blood cultures were negative thereafter and remained so until the 14th day at which time the experiment was discontinued. A dose of 1 ml. of living culture proved fatal to each of 2 lambs although one of these died after the unusually long period of 68 hours and showed an atypical post-mortem appearance which is described later. The heat-killed culture possessed considerable toxicity and even 10 ml. killed one of 2 lambs inoculated; however, 1 ml. contained in the standard dose volume of 100 ml. produced no observable effect. The macroscopic lesions produced by living and heat-killed culture were indistinguishable and closely resembled those described by Stamp *et al.* (1955). Lungs were congested, haemorrhagic and oedematous and clear, amber pleural exudate was frequently found. Exudative pericarditis and haemorrhages on the subepicardial and endocardial surfaces of the heart were almost constant findings. The abomasal mucosa commonly showed intense, haemorrhagic inflammation, sometimes resulting in a livid, blackish-purple colouration. Relatively slight intestinal inflammation and small amounts of peritoneal exudate were often present. Cultures of spleen tissue from lambs which died after receiving lethal doses of heat-killed organisms were invariably negative. Counts of viable organisms in the tissues of lambs which died from doses of living bacteria yielded results which, with one exception, did not differ significantly from those recorded in Table 3. The exception was the lamb, mentioned earlier, which died 68 hours after receiving a dose of 1 ml. of living culture. At autopsy the most obvious lesion shown by this animal was a large consolidated area involving the anterior third of each lung. Counts of viable organisms in tissues gave the results shown below.

Consolidated lung	222×10^6 per g.
Non-consolidated lung	7×10^6 ,, ,,
Liver	11×10^6 ,, ,,
Spleen	25×10^6 ,, ,,

DISCUSSION

The purpose of the work recorded in this paper is to throw more light on the pathogenicity of *Past. haemolytica,* Type T, in naturally occurring and experimental septicaemia of lambs as described by Stamp *et al.* (1955). Observations have shown that the fatal diseases which can be produced by natural infection, by the intravenous injection of living organisms and by the intravenous injection of heat-killed organisms are all very similar. The most obvious symptoms are respiratory distress, diarrhoea and rapid prostration, while the post-mortem appearance is characterised by congestion, haemorrhage and oedema. This corresponds, according to Topley and Wilson (1955), with the type of disease which is produced by toxic concentrations of Gram-negative organisms.

The experimental strain of *Past. haemolytica* was derived from one of three lambs, all of which were examined immediately after death and whose tissues were shown to contain extremely large numbers of bacteria. That these numbers of bacteria contained more than sufficient toxic material to bring about death was strongly suggested by an experiment in which considerably smaller numbers of heat-killed organisms proved fatal when injected by the intravenous route. Presumably in the natural cases a large increase in the number of organisms occurred between the time at which a lethal level had been reached and the time of death. Theoretically such an increase could occur relatively quickly since the lethal level must in itself represent a large number of organisms and consequently even a few succeeding bacterial generations would result in enormous proliferation. The same concept could account for the sudden onset of symptoms and rapid death which is seen in both the natural and experimental disease. In one of the experiments described 1 ml. of heat-sterilised broth culture produced no observable toxic symptoms in 2 lambs, 10 ml. killed 1 of 2 lambs and 50 ml. killed each of 2 lambs. In terms of bacterial generations the organisms contained in 1 ml. of culture are not far removed from those in 10 ml., 50 ml. or more, and in the same way a sub-toxic dose of living bacteria may not be far removed, both in size and in time, from the lethal dose.

The natural disease occurs most frequently in lambs between 6 and 9 months of age and is rarely seen in animals more than a year old. These facts, together with the demonstration by Smith (1960) of a marked relationship between age and resistance to infection with *Past. haemolytica* Type A, suggested the experiment in which a freshly isolated Type T strain was titrated intravenously for virulence in adults and in 7-month old lambs. Using 100-fold dose dilutions no difference in susceptibility between lambs and adults could be shown in terms of death and survival. A definite difference was revealed however by means of blood cultures which showed that the bacteria were removed more quickly from the peripheral circulation of adults than of lambs. The experiment also showed that while the large dose of 10 ml. of 5-hour broth culture ($8,200 \times 10^6$ viable organisms) killed all animals inoculated, one hundredth of this dose failed to produce any deaths. A subsequent experiment using lambs showed that the lethal dose of dead organisms constituted only a low multiple of the lethal dose of living organisms. Even 10 ml. of sterilised culture, contained in the standard dose volume of 100 ml. killed one of 2 lambs inoculated intravenously. In view of this finding it seems reasonable to suppose that the large doses used by earlier workers to produce fatal infections in lambs were either very close to a toxic level or perhaps on occasions even toxic in themselves without further multiplication. That the tissue suspensions which constituted certain of the inocula used by Stamp *et al.* (1955) also contained large numbers of bacteria is likely in view of the high counts of viable bacteria in the tissues of natural cases recorded in Table 1. It should be emphasised however that the doses used by Stamp *et al.* almost exactly reproduced the natural disease. This is confirmed by the work of Biberstein and Kennedy (1959) and also by the present investigations.

It is inconceivable that infection by such overwhelming numbers of organisms could occur in the field and the mechanism of infection in the natural disease is still obscure. Nevertheless, the finding that even small doses of *Past. haemolytica,* Type T, inoculated intravenously into lambs can result in a bacteraemia which in some instances may persist over a period of several days is possibly of importance.

CONCLUSIONS

The tissues of 6-month-old lambs which died from natural septicaemic infections of *Past. haemolytica,* Type T, were shown to contain exceedingly large numbers of viable bacteria.

Very high intravenous doses of a freshly isolated T strain produced death in 7-month-old lambs and adults, but smaller doses were not fatal. Smaller doses did however demonstrate a relationship between age and resistance in that bacteria were eliminated more rapidly from the peripheral circulation of adults than of lambs.

The lethal dose of heat-killed broth culture for lambs by intravenous inoculation was only a low multiple of the lethal dose of living culture.

ACKNOWLEDGMENTS

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ANAESTHESIA IN THE FOWL

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INTRODUCTION

Anaesthesia of the domestic fowl has presented many difficulties which have not been satisfactorily surmounted. The investigation described in this paper was undertaken to find a method which would provide deep, safe, general anaesthesia.

There are numerous reports on the use of ether and chloroform (Pearl and Surface, 1919; Kaupp, 1923; Mayrs, 1924; Gaddum, 1928; Hole, 1933; Lee, 1953 and Gandal, 1956). The toxicity of the volatile anaesthetics was stressed by all but Gandal (1956) who showed that ether may be safely used if administered intermittently for short periods.

Chloral hydrate was given intravenously by Hole (1933) and Schwarte (1952), and orally by Lee (1953), King and Biggs (1957) and Wright (1958). Discrepancies exist among the reports on the depth and duration of anaesthesia.

Fretz (1932) injected sodium amylobarbitone (sodium amytal) intravenously but Lee (1953) did not recommend the drug and considered that sodium thiopentone (pentothal) was also unsatisfactory. Sodium pentobarbitone has been used by numerous workers, including Warren and Scott (1935), Graham (1939), Blount (1947), Gordeuk and Grundy (1950), Durant (1953), Cragg, Evans and Hamlyn (1954), Gandal (1956), Shirley and Nalbandov (1956), King and Biggs (1957), Worden and Lane Petter (1957), Wright (1957) and Newman and Taylor (1958). Most workers pointed out that the anaesthetic dose was close to the lethal one but only a few advised against its use. Combuthal, which is a mixture of sodium thiopentone and sodium pentobarbitone (3:1), was recommended by Sanger and Smith (1957) and Church (1957). Phenobarbitone was given intramuscularly by Coon (1939) and King and Biggs (1957), and intraperitoneally by Harvey, Copen, Eskelson, Graff, Paulsen and Rasmussen (1954). Coon, and Harvey *et al.* reported satisfactory results but King and Biggs found the drug erratic in action. While Koppanyi and Murphy (1933) found sodium barbitone too toxic for use as an anaesthetic, Harvey *et al.* (1954), and Rennick, Calhoon, Gandia and Moe (1954) obtained satisfactory results.

Equithesin, which is a mixture of chloral hydrate, sodium pentobarbitone, and magnesium sulphate, was given intramuscularly by Gandal (1956), and Sanger and Smith (1957). They found it to be a satisfactory general anaesthetic in the fowl but there was some discrepancy in the duration of anaesthesia.

Previous reports of anaesthesia in the fowl have presented conflicting claims and in many instances details of technique have been omitted. For instance, the method of assessing the depth of anaesthesia is rarely mentioned, whilst the duration of anaesthesia and fatalities are frequently unrecorded. These discrepancies and omissions suggested a re-investigation of some of the methods used.

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MATERIALS AND METHODS

A variety of anaesthetics and methods were investigated in a series of preliminary tests and from these the most promising were selected for more detailed examination.

The birds used were Thornber's '505' Hybrids of both sexes and 4 to 12 months of age. Many of them were anaesthetised on more than one occasion, with an interval of at least 14 days between successive experiments. During anaesthesia the environmental temperature was maintained at approximately 70°F. Intravenous injections were made into a wing vein and intramuscular injections were made deeply into the pectoral muscles. The depth of anaesthesia was assessed by the response of the bird to pinching the comb and interdigital web. Complete lack of response to stimulation of both these structures was termed deep anaesthesia, while slight response to either of them was termed light anaesthesia. In no birds which survived the anaesthetic was there loss of the corneal reflex.

In each experiment observations were recorded on the weight and age of bird, the method of administration of anaesthetic, the induction time, the depth and duration of anaesthesia, the recovery time, the mortality, the total amount of anaesthetic used, and the rectal temperature and respiratory rate before and during anaesthesia.

RESULTS

Preliminary Trials

In these trials each anaesthetic was tested on at least 3 birds. There were 3 main reasons for rejecting an anaesthetic, (1) induction time more than one hour (2) anaesthesia less than ten minutes, (3) mortality greater than 50 per cent. In Table 1 the rejected anaesthetics are classified accordingly.

TABLE 1

ANAESTHETIC AGENTS REJECTED AFTER PRELIMINARY TRIAL

Induction time more than 1 hour.
20 per cent solution sodium phenobarbitone[1] 180 mg/kg i.p.
20 per cent solution sodium phenobarbitone 200 mg/kg i.m.

Period of anaesthesia less than 10 minutes.
Nitrous oxide by inhalation; no anaesthesia obtained.
5 per cent solution sodium thiopentone i.v. until anaesthesia obtained.
20 per cent solution sodium phenobarbitone with 6 per cent sodium pentobarbitone in the ratio of 3:1 by weight, i.v. until anaesthesia obtained.
Equithesin[2] 2·5 to 3·0 ml/kg. i.m.
Equithesin 5 to 6·0 ml/kg. i.m.
Equithesin 5 to 6·0 ml/kg. i.v. until anaesthesia obtained.
C.M.1[3] i.m. followed by 6 per cent sodium pentobarbitone i.v. until anaesthesia obtained.
Combuthal[4] i.v. until anaesthesia obtained.
Chlorpromazine hydrochloride[5] 2·5 per cent, 1·5 to 17·0 mg/kg. i.m.
Chlorpromazine hydrochloride 2·5 per cent, 1·5 to 17·0 mg/kg i.m. followed by 6 per cent sodium pentobarbitone i.v. until anaesthesia obtained.

Mortality greater than 50 per cent.
20 per cent chloral hydrate i.v. until anaesthesia obtained
5 per cent chloral hydrate i.v. ,, ,, ,,
6 per cent sodium pentobarbitone 120 mg/kg. i.m.
20 per cent sodium phenobarbitone 200 mg/kg. i.m.

1. Luminal (Bayer) containing 0·2 g. phenobarbitone in each 1 ml. of N-methyl acetamide.
2. Equithesin (Jensen-Salsbery Labs. Kansas City, U.S.A.) contains 4·26 per cent chloral hydrate, 0·96 per cent sodium pentobarbitone and 2·12 per cent magnesium sulphate in an aqueous solution of 20 per cent propylene glycol with 9·5 per cent ethanol.
3. C.M.1 is a solution of chloral hydrate and magnesium sulphate; its formula is given on page 442.
4. Combuthal (Abbott Labs.) is an aqueous solution containing 2·25 per cent sodium thiopentone and 0·75 per cent sodium pentobarbitone.
5. Largactil (May & Baker Ltd.).

Selected Anaesthetics

*Ether.** Two groups of birds numbering 8 and 12 were used. Each bird was given 0·5 mg. of atropine sulphate subcutaneously one hour before induction of anaesthesia. Anaesthesia was induced by holding the head of the bird in a beaker of 8 to 10 cm. diameter containing cotton wool soaked in ether. From time to time during anaesthesia the respirations became slow and often very shallow and at these times the beaker was removed, being replaced only when breathing became normal.

TABLE 2

ANAESTHESIA WITH ETHER

Total number of birds	*Deaths*		*Survivors*			
				Weight	*Deep anaesthesia*	
	No.	*Per cent*	*No.*	*(Kg.)*	*No.*	*Duration (mins.)*
8	2	25	6	1·54	2	30
				± 0·3	3	40
				1·19—1·96	1	60
12	2	17	10	1·71	4	20
				± 0·38	2	30
				1·28—2·32	1	40
					3	60

Mean, mean deviation and range are given for weights.

Induction took 2 to 4 minutes and there was little or no struggling. Birds were anaesthetised for 20, 30, 40 or 60 minutes after which the anaesthetic was removed and the birds allowed to recover (Table 2). Breathing ceased in 3 birds from the first group and 5 birds from the second group and artificial respiration was attempted by passing a 'Polythene' tube down the trachea, aspirating from the lungs and air-sacs and then blowing down the tube. Four of the 8 birds so treated recovered and were then anaesthetised for further periods of 20 to 40 minutes without incident. Anaesthetised birds recovered within 30 minutes of removal of the anaesthetic.

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* Aether anaesthesticus B.P.

*Sodium pentobarbitone i.v.** A 6 per cent solution of sodium pentobarbitone in 10 per cent propylene glycol was given by slow intravenous injections at a rate of approximately 0·1 ml/ minute to 10 birds. Injection was stopped when deep anaesthesia had been induced. The results are summarised in Table 3 which shows that the birds weighed 1·19 to 2·15 kg. and were given 44·2 mg/kg. of pentobarbitone. These doses gave a period of 11 minutes deep anaesthesia and about 30 minutes total anaesthesia. Preliminary tests indicated that the rate of injection was very important, since if it were too rapid the birds died from respiratory failure during induction. All 10 birds recovered within 8 to 12 hours.

Phenobarbitone i.m. and pentobarbitone i.v. The results of preliminary tests suggested that anaesthesia induced by the intravenous injection of pentobarbitone might be prolonged if an intramuscular injection of phenobarbitone were also given. Three doses of phenobarbitone, 150, 200 and 250 mg/kg. were selected and pentobarbitone was given by slow injection to a total of 44 birds until deep anaesthesia was obtained. A dose of 150 mg/kg. of phenobarbitone given intramuscularly, followed immediately by pentobarbitone intravenously produced deep anaesthesia of less than 30 minutes duration, whilst larger doses caused a high mortality (Table 4). An unfavourable feature of this method was the occurrence of partial recovery soon after induction. This lasted as long as one hour in some birds and was followed by a second period of deep anaesthesia.

TABLE 3

ANAESTHESIA WITH SODIUM PENTOBARBITONE I.V.

Ten birds, all survived

Weight (*kg.*)	*Dose* (*mg/kg.*)	*Induction time* (*min.*)	*Duration of anaesthesia* (*min.*)	
			Deep	*Total*
1·53	44·2	14·9	11	30·2
± 0·23	± 7·2	± 6·1	± 4·4	± 9·6
1·19—2·15	31—59	5—31	4—25	12—60

Mean, mean deviation and range are given.

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* Nembutal, Veterinary (Abbot): a 6 per cent. solution in 10 per cent. propyleen glycol.

TABLE 4

ANAESTHESIA WITH SODIUM PHENOBARBITONE I.M. AND SODIUM PENTOBARBITONE I.V.

Total number of birds	*Deaths*		*Survivors*					
							Deeply anaesthetised for more than 30 min.	
	No.	*Per cent*	*No.*	*Weight (k.g.)*	*Pheno-barbitone (mg/kg.)*	*Pento-barbitone (mg/kg.)*	*No.*	*per cent*
10	3	30	7	1·67	150	38·1	0	0
				± 0·45		± 6.33		
				0·91—2·87		27·0—49·0		
29	15	52	14	1·38	200	32·0	7	50
				± 0·32		± 3·48		
				0·88—2·58		23·0—48·0		
5	4	80	1	1·42	250	32·6	0	0

Mean, mean deviation and range are given for weights of birds and dose of pentobarbitone.

C.M.2 and *C.M.3 i.m. and pentobarbitone i.v.* In the preliminary trials, Equithesin given both intravenously and intramuscularly proved to be unsatisfactory, but more promising results were obtained when the chloral hydrate and magnesium sulphate constituents (C.M.) were given intramuscularly, followed by pentobarbitone intravenously. Mixtures termed C.M.1, 2 and 3, the formulae for which are given below, were prepared. C.M.1 was discarded in the preliminary trials but C.M.2 was found to merit further investigation and a modification of it (C.M.3) was also used.

C.M.1	Chloral hydrate	42·6 g
	Magnesium sulphate	21·2 g
	Propylene glycol	200 ml
	Ethanol	100 ml
	Distilled water to	1000 ml

This formula is the same as that for Equithesin but without the sodium pentobarbitone.

C.M.2	Chloral hydrate	170·4 g
	Magnesium sulphate	84·8 g

Propylene glycol, ethanol, and distilled water as in C.M.1.

C.M.3 Similar to C.M.2 but without propylene glycol or ethanol and made up to 1000 ml. with distilled water.

A total of 42 birds in 4 groups were used (Table 5). Thirty-two birds in groups 1, 2 and 3 were given C.M.2 and 10 birds in group 4 were given C.M.3. The dose of 1·65 ml/kg. was given to each bird by injecting half the solution into the left and half into the right pectoral muscles in an attempt to minimize muscle damage. Immediately following the injection of C.M. pentobarbitone was given until anaesthesia was induced. To determine the effect of C.M. on the pectoral muscles, 12 birds in 3 groups of four were injected with 1·65 ml/kg. of C.M.2, C.M.3 and distilled water respectively. One bird from each group was killed on the 3rd, 11th, 17th and 24th day after injection. At autopsy the general appearance of the muscle was noted and a portion fixed in 10 per cent formalin or Bouin's fluid for histological examination.

There was no detectable difference between the anaesthetic effects of C.M.2 and C.M.3 and birds of both sexes were similarly affected. The mortality rate was 7 per cent and the surviving 39 birds were used to evaluate the anaesthetic. The 3 birds which died were males of above average weight and it may be that such birds require less than the average dose for lighter birds.

Anaesthesia was induced approximately 10 minutes after the birds had been given 1·65 ml/kg. of C.M.2 or 3 and an average of 27 mg/kg. of pentobarbitone. Deep anaesthesia lasted for about 60 minutes, although there was a wide variation from 10 to 150 minutes (Table 5). From Figure 1 and Table 5 it will be seen that all the birds were anaesthetised for at least 10 minutes and that approximately 75 per cent were deeply anaesthetised for about 30 to 60 minutes. Only about 15 per cent were deeply anaesthetised

TABLE 5

ANAESTHESIA WITH 1·65 ML/KG. OF CM2 OR CM3 I.M. AND PENTOBARBITONE I.V.

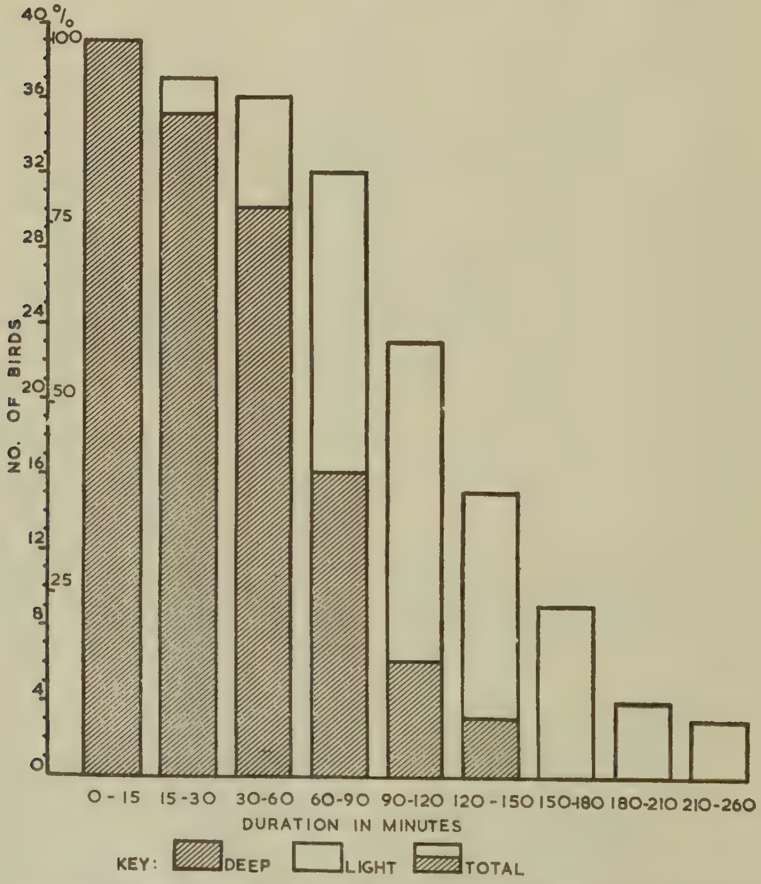
Total number of birds		*Deaths*		*Survivors*					
								Duration of anaesthesia (min.)	
Group	*No.*	*No.*	*Per cent*	*No.*	*Weight (kg.)*	*Pentobarbitone (mg/kg.)*	*Induction time (min.)*	*Deep*	*Total*
1 (CM 2)	10	0	0	10	1·283 ± 0·211 0·850—2·000	21·6 ± 5·2 11—38	10·5 ± 2·0 7—14	28 ± 17 10—63	75 ± 39 10—175
2 (CM 2)	14	1	7	13	1·330 ± 0·154 0·955—1·585	28·2 ± 3·8 10—38	9·5 ± 2·0 6—15	56 ± 32 10—147	100 ± 38 24—167
3 (CM 2)	8	0	0	8	1·524 ± 0·288 0·940—2·120	28·5 ± 4·0 21—34	10·0 ± 3·0 6·22	78 ± 27 32—150	102 ± 32 32—170
4 (CM 3)	10	2	20	8	1·606 ± 0·339 1·015—2·250	27·0 ± 6·0 16—38	8·5 ± 3·5 5—16	63 ± 32 25—136	169 ± 65 31—260
TOTAL	42	3	7	39	1·415 ± 0·249 0·850—2·250	27·0 ± 5·8 11—38	9·6 ± 2·7 5—22	59 ± 29 10—150	108 ± 48 10—260

Mean, mean deviation and range are given for weight of bird, dose of pentobarbitone, induction time and duration of anaesthesia.

for more than 90 minutes, and none for more than 150 minutes. Total anaesthesia lasted for an average of 108 minutes but there was a wide variation from 10 to 260 minutes. The duration of total anaesthesia was 30 to 60 minutes in about 90 per cent of the birds, about 25 per cent were still lightly anaesthetised after 180 minutes and none was lightly anaesthetised for more than 260 minutes. Most of the birds had recovered about 24 hours after the end of anaesthesia, although a few required longer.

Effects of C.M.2 and 3 on the pectoral muscles (Figs. 2,3,4) C.M.2 and 3 produced similar gross and microscopic changes in the pectoral muscle. The birds showed no clinical evidence of muscular injury and appeared to be in normal condition as judged by weight, feeding and general appearance. The muscular tissue of birds which were examined 3 days after injection with distilled water, showed slight damage and no further examinations were made in this group.

Fig. 1



Histogram of duration of deep, light and total anaesthesia in 39 birds given C.M.2 or C.M.3 followed by pentobarbitone.

Three days after injection with C.M.2 or 3 there was oedema and swelling of the pectoral muscles. An ellipsoidal area of haemorrhage and necrotic tissue, of maximum dimensions 0·5 x 1·0 x 3·0 cm., was surrounded by a more extensive zone of pale muscle covering an area about 6 x 2 x 1·5 cm. Microscopical examination revealed muscular degeneration with extravasated blood and thrombi, the degeneration being most severe at the centre of the lesion. There was extensive phagocytosis of necrotic muscle, macrophages and foreign-body giant cells being abundant. In some areas the endomysium showed a reaction to the injection by proliferation of macrophages. Although granulocytes and lymphocytes were present, leukocytes were not abundant in the connective tissue. Muscle which was examined 11 and 17 days after injection showed a marked decrease in the extent of the lesions which, however, were still visible at 24 days. The histological appearance was generally similar to that seen at 3 days, although there was a progressive decrease in the necrotic tissue and an increase in connective tissue. Some regeneration of muscle was observed in the lesions at 17 days.

DISCUSSION

In a discussion of anaesthesia in the fowl it is essential to know the criteria by which anaesthesia is assessed. For this reason it is only possible to compare our results with those reports in which the criteria are indicated. Warren and Scott (1935), Blount (1947), Gordeuk and Grundy (1950) and King and Biggs (1957) each indicated that the only satisfactory guide to anaesthesia was the failure of the bird to respond to surgical interference. Hole (1933) used the loss of the corneal reflex as a guide to deep anaesthesia, whilst Cragg *et al* (1954) judged by the respiratory rate. In our hands neither of these two methods proved entirely satisfactory. For instance, there was no loss of the corneal reflex in any of our birds which recovered from the anaesthetics; when the reflex was absent the bird invariably died. Again, we found no correlation between the respiratory rate and the depth of anaesthesia. The only indication of anaesthesia that we found to be satisfactory was the loss of the response to pinching the interdigital web and the comb. The absence of both these reflexes indicated complete lack of response to most stimuli in the anaesthetised bird, but the corneal reflex was always present and stimulation of the epiglottis sometimes evoked a response. During operations for neurohypophysectomy which lasted about two hours, undertaken by one of us (A.W.), the comb and foot reflexes were found to be admirable indications of anaesthesia, the birds remaining completely unresponsive to such strong stimuli as the use of the electrocautery.

It is possible that other factors, including age and weight of bird, breed and pre-induction starvation may affect the depth and duration of anaesthesia. It is therefore wise to make some preliminary tests to ascertain whether the doses advocated for a particular breed are applicable to another. Pre-induction fasting is suggested to prevent the regurgitation of food and water from the crop.

The volatile anaesthetics have, in general, been rejected on account of their toxicity, which is probably associated with their accumulation in the air-sacs and lungs. We found ether to be satisfactory and safe provided that the respiration of the bird is continuously observed. A second operator is therefore essential and our fatalities occurred when this was not practised. For ease of administration an aspirator-pump in a circuit which delivers either air, ether, or CO_2 when required, is easily constructed. Fine control of the amount of ether is readily made and resuscitation of the bird is facilitated by allowing CO_2 to enter the circuit when necessary. Ether has the great advantage of short induction and recovery times.

Although pentobarbitone has been widely used in anaesthesia of the fowl it cannot be considered a good general anaesthetic. The considerable divergence of opinion found in earlier reports on the value of pentobarbitone is perhaps associated with the different depths of anaesthesia required by various authors. We agree with earlier workers that the anaesthetic dose is close to the lethal dose but that the drug may be useful where anaesthesia of short duration is needed. In combination with pentobarbitone, phenobarbitone was not satisfactory. The mortality rate was high and at lower dosage the duration of anaesthesia was short.

Equithesin has been used to a limited extent during the surgery of chickens and turkeys by Gandal (1956) and Sanger and Smith (1957). When Equithesin was administered in the doses recommended by Gandal we failed to induce anaesthesia, and when the dose was doubled an extremely long induction period was followed by a a very short period of anaesthesia. However, by injecting two of the components of Equithesin, chloral hydrate and magnesium sulphate, in one solution (C.M.) intramuscularly, and the pentobarbitone separately intravenously, good results were obtained. The quantity of chloral hydrate and magnesium sulphate required to induce deep anaesthesia was found to be approximately 2·5 times that recommended by Gadal in using Equithesin. When propylene glycol and ethanol were omitted from the formula (C.M.3) equally good results were obtained. Compared with Equithesin the induction period was greatly reduced by injecting pentobarbitone directly into the wing vein. C.M.2 or 3 followed by pentobarbitone very rapidly induces a period of anaesthesia sufficient to enable operations of long duration to be completed. The method is simple, the mortality rate is low and the birds need a minimum of surveillance. On the other hand there is some muscular damage and the duration of anaesthesia is variable. However, the general health of the bird is not affected, the area of damage to the pectoral muscles is small in relation to the muscle mass and after three weeks the lesion has substantially healed. It is therefore reasonable to infer that no long-standing or permanent damage is incurred and it is interesting to note that birds showed no clinical evidence of the muscle injury. Sanger and Smith (1957) reported that Combuthal and Equithesin caused damage to the pectoral muscles of one-day, and five-day old chickens and 3·2 to 6 kg. turkeys. Muscular necrosis occurred, but after 14 days the lesions had disappeared. It is possible, therefore, that the higher concentrations of chloral hydrate and magnesium sulphate in C.M.2 and 3 in comparison with Equithesin produced more severe muscular damage. The variation in the duration of anaesthesia with C.M. was a puzzling feature of the method and we were not able to resolve the problem. However, examination of pectoral muscle at autopsy showed that in some birds the injection had been made not into, but between, the superfiical and deep pectoral muscles, which may have affected the absorption of the C.M.

The completely unsatisfactory results obtained using Chlorpromazine are in contrast to its usefulness in mammalian sedation. It is probable that the action of the drug in the fowl is different from that in mammals.

Although the respiratory rate and temperature were recorded throughout the experiments, no general conclusions could be drawn. There was usually, however, a rapid variable decline in temperature from the normal average of 107·7°F. during induction and a very gradual return to normal. Neither the body temperature nor the respiratory rate could be correlated with the depth or duration of anaesthesia in any of our experiments.

In conclusion, it can be said that no method of anaesthesia which we tried was completely satisfactory. Little is known about the action of anaesthetics in birds and the methods are therefore empirical. It is suggested that fundamental work on the action of anaesthetics in the fowl is essential before an accurate appreciation of the birds' response can be obtained and a satisfactory anaesthetic formulated.

CONCLUSIONS

Preliminary trials of 12 methods of anaesthesia in the fowl led to 4 being selected as most suitable:—ether by inhalation; pentobarbitone intravenously; phenobarbitone intramuscularly followed by pentobarbitone intravenously; and a mixture of chloral hydrate and magnesium sulphate (C.M.) given intramuscularly and followed at once by pentobarbitone intravenously.

Ether was reasonably satisfactory and produced deep anaesthesia with rapid induction and recovery, but an assistant is necessary for the continuous observation of respiration and for the control of the depth of anaesthesia.

Pentobarbitone intravenously in small doses induced a short period of anaesthesia; higher doses caused a high mortality.

C.M. intramuscularly, followed at once by pentobarbitone intravenously, was the most satisfactory anaesthetic and was both convenient and safe. In 30 out of 39 birds the method produced deep anaesthesia of 30 to 60 minutes duration after an induction period of 10 minutes. The intramuscular injection caused slight muscle damage which did not interfere with the health of the bird.

No anaesthetic was found which gave prolonged anaesthesia in all cases and fundamental work on anaesthesia in the fowl is required.

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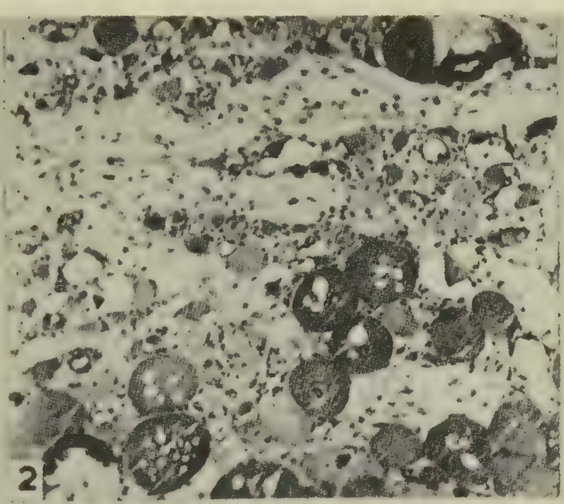

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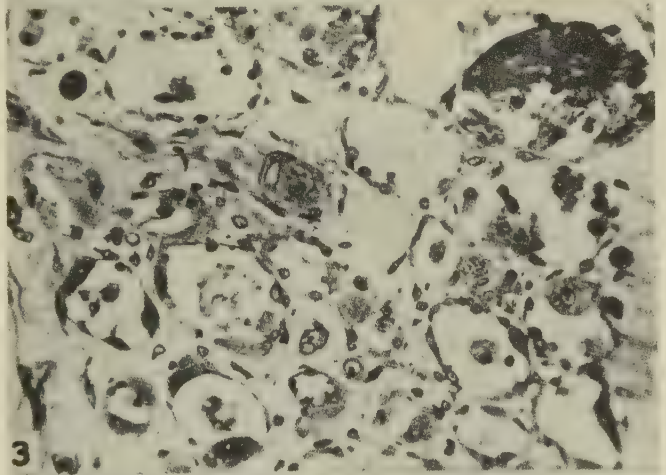

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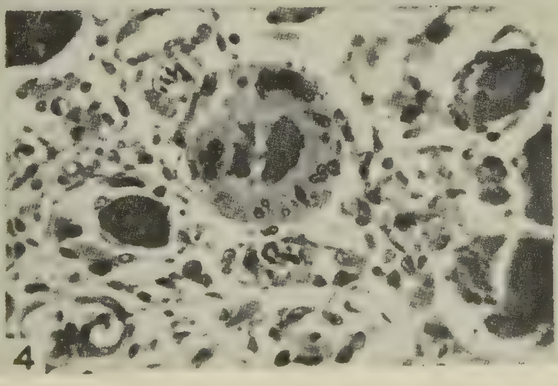

4

LEGENDS TO ILLUSTRATIONS

Figs. 2, 3, 4 are photomicrographs of the pectoral muscle of birds killed 3 days after injection of C.M.2. Tissues were fixed in Bouin's fluid and stained with Harris's haematoxylin and eosin.

Fig. 2. Part of necrotic muscle. Degenerate muscle fibres in T.S. at bottom of field showing vacuoles. The centre of the field shows an area from which most of the muscle fibres have disappeared leaving the endomysium, which shows proliferation of macrophages. Acutely degenerate muscle fibre at top right. × 120.

Fig. 3. Different bird, similar region. Shows proliferation of macrophages from endomysium; spaces containing free macrophages and some muscle debris; macrophages and connective tissue cells in interstitial tissues; severely degenerated muscle fibre at top right. × 340.

Fig. 4. Same tissue as Fig. 3. Foreign-body giant cell surrounding a necrotic muscle fibre. Other degenerate muscle fibres are seen. × 340.

PANCREATIC INSULOMA WITH CLINICAL MANIFESTATIONS IN A DOG

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INTRODUCTION

An intriguing group of neoplasms are those which arise in endocrine tissue and which through production of specific hormones have profound effects upon the body quite beyond those of tumours in general. Pancreatic insuloma in the dog belongs to this select group. Of the fourteen reported cases in which the diagnosis seems reasonably certain, Cello and Kennedy (1957) observed three, McGrath (1956) four, and six were seen in Stockholm (Hansen, 1949; Hansen and Krook, 1958). The detection of yet another insuloma in Stockholm suggests that the tumour is not uncommon but that its clinical manifestations may not be fully appreciated.

CASE REPORT

Clinical Manifestations

An eight-year-old Boxer bitch had been subject to episodes of unconsciousness for a period of one year. The intervals between episodes had gradually decreased and during the last month unconsciousness, often in conjunction with exercise, occurred practically every day. The first sign of impending unconsciousness noticed by the owner was swaying of the hind-quarters rapidly followed by flaccid collapse. During unconsciousness, which generally persisted about a minute, the bitch lay on her side with scarcely perceptible tonic extension of the limbs but no other convulsive muscular activity. Upon recovery the animal was subdued and weak and went straight to her sleeping basket. The last episode of unconsciousness occurred two days before the bitch was killed at the owner's request.

The only other abnormality reported by the owner was that the bitch was exceedingly thirsty during the last weeks. Oestrus last occurred a month before death; the bitch had whelped once, four years previously.

Post-mortem Examination

The autopsy was carried out three hours after death. The bitch weighed 29 kg, was muscular and had a moderate deposition of fat.

The pancreatic insular tumour was only 5 mm in diameter and was situated in the middle of the free border of the duodenal portion of the pancreas. The tumour tissue was whitish and fairly firm and, in fact, could be readily distinguished from the adjacent pancreatic tissue only after fixation. The pancreatic and portal lymph nodes were of normal size.

Other autopsy findings were moderate, focal renal scarring (parathyroids not enlarged), slight focal hyaline vascular degeneration in the myocardium, lungs and leptomeninges, a solitary splenic lymphoma, and the ovarian and uterine changes of metoestrus. The vertebral column and long bones were sawn longitudinally.

Microscopical Examination

The tumour lies immediately under the pancreatic capsule and is clearly separated from the parenchyma except for some small islands of acinar tissue towards the edges (Fig. 1). Internally it is bounded in part by a well-developed connective tissue capsule; in places, however, little more than a few reticulin strands separate the tumour from acinar tissue. There are no signs of infiltration and only slight compression of the adjacent pancreatic parenchyma. Apart from strands of connective tissue originating from the internal capsule of the tumour and the supporting tissues about blood vessels, the tumour tissue has a sparse stroma, not much more than a network of small blood vessels and capillaries. The irregular spaces between the blood vessels, most of which are fairly small although some are large, are filled with tumour cells, tightly packed in places and loosely in others.

The amount and appearance of the cytoplasm of these cells varies somewhat from field to field while the relatively large nuclei, which are rounded or oval, vesicular, and with distinct nucleoli and fine chromatin clumps, are fairly uniform. Over large areas the cells are obviously voluminous, but the cytoplasm is evident only as a narrow rim marking what is presumably the cell border. No fat could be demonstrated in these cells. Among the large, heavily vacuolated cells can be found dense groups of smaller cells with a finely granular, slightly basophilic cytoplasm, indistinguishable from islet cells in the adjacent normal pancreatic tissue (Figs. 2 and 3). With Gömöri's chrome-haematoxylin-phloxine stain employed on formalin-fixed tissue as described by Hansen (1949), i.e. refixation of the section for 24-hours in Bouin's fixative before staining, the cytoplasmic granules in some cells stain deep blue as do those in β-cells in the adjacent pancreatic parenchyma (Fig. 3). Within the tumour, the few cells of this type with a blue granular cytoplasm are generally arranged as "rosettes" in one or several layers about a central space (Fig. 2). By no means all cells with this arrangement and with a granular cytoplasm take the stain. For cells with stainable granules, however, staining intensity is uniform throughout the tumour.

Within the tumour, small ducts surrounded by a few strands of collagen and resembling the fine extra-acinar ducts of normal pancreatic tissue are encountered to the same extent as in pancreatic tissue taken at a distance from the tumour, from one to three per high-power field.

In the pancreatic tissue adjacent to the tumour there is an obvious increase in the size of Langerhan's islets in comparison with more distant parts of the pancreas. The proportion of β-cells, i.e. those with a blue granular cytoplasm with Gömöri's chrome-haematoxylin-phloxine stain, is somewhat more than half in islets throughout the pancreas.

No nervous tissue could be identified in haematoxylin and eosin stained sections of the tumour. No attempt was made to stain the so-called silver cells (Hultquist, 1946).

DISCUSSION

The diagnosis of pancreatic insuloma in this instance is based upon the site of the tumour, the general appearance of the tumour cells, their arrangement in clusters throughout a sparse but highly vascular stroma and the demonstration of cells with staining characteristics resembling those of insular β-cells. Even in the absence of blood glucose determinations, the anamnesis of recurring episodes of unconsciousness and the lack of other lesions which could reasonably account for this justify the inclusion of the tumour among the active representatives of its type. Full verification, of course, demands the demonstration of depressed blood sugar levels, an abnormal glucose tolerance curve, the elimination of other potential causes of hypoglycaemia and the demonstration of insulin activity in the tumour tissue.

Most of the previous reports of canine insuloma contain adequate summaries of what is known about this tumour in dogs and human beings. Here, only the main features need to be recalled. The age of affected dogs falls within the expected range. Various breeds have been represented, the Boxer most often. Boxers tend to be over-represented in statistical surveys of canine tumours, at least in Stockholm-gliomata (Hjärre, 1938), carcinomata (Krook, 1954), heart-base tumours (Nilsson, 1955), and mastocytoma (Larsson, 1956, 1957). The major features are summarised in Table 1.

Basically, of course, the clinical signs are those of hyperinsulism with, in dogs, recurring episodes of extreme fatigue, often with motor disturbances, associated with exercise or fasting. In human beings, subjective symptoms such as uneasiness, irritability, confusion and so forth seem to be usual in the early stages while full unconsiousness appears as a fairly late manifestation (Himwich, 1944).

The characteristic clinical feature of pancreatic insuloma is the intermittency in obvious signs concomitant with depressed but more or less stable blood glucose levels. A fully satisfactory explanation has not been given. Fabrykant and Bruger (1948) have proposed what they call the "dynamic concept of cerebral carbohydrate metabolism." Morphological studies of the tumours have given no clues (O'Leary and Womack, 1934; Duff, 1942). Paradoxically, the blood glucose level may be no greater during intervals between episodes of unconsciousness, and fasting an animal known to have an insuloma and often subject to alarming clinical signs may be insufficient to induce the typical signs (Cello and Kennedy, 1957).

Pancreatic insulomas are not necessarily functionally active in human beings and dogs. Only one example of non-functional insuloma has been described in a dog (Hansen, 1949). Hansen has kindly given me details of two additional unpublished cases of inactive canine insuloma. Both had produced metastases in the regional lymph nodes and liver.

Neither the duration of clinical signs nor the blood glucose level appear to be reliable guides for estimating the presence or absence of metastases (Table 1). The proportion of metastasising tumours is undoubtedly artificially great since apart from the tumours which have been recognised clinically, the chances of finding these often inconspicuous tumours are clearly much greater when there are obvious metastases.

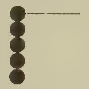
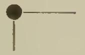
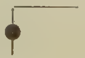
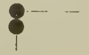
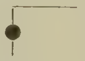
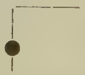
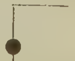
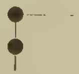
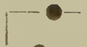
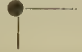
Many of the previous reports of canine insuloma were not primarily morphological descriptions; nevertheless enough details can be garnered to give a reasonably valid general impression. Although usually single, multiple pancreatic tumours were seen in three dogs. Most of the tumours were situated in the duodenal portion of the pancreas (Table 1). There is no obvious reason for this—the duodenal and the splenic portion are approximately the same size and, paradoxically, islets are more numerous in the splenic branch than in the duodenal (Hjärre, 1927). Size has ranged from about 5 mm in diameter to 3 × 2 × 2 cm. Colour has generally been described as greyish white or greyish purple. Macroscopically at least, the tumours have appeared to be well encapsulated. An abundant and heavy stroma has been described in most instances, but in parts of Case I in Hansen's series of tumours (1949) and throughout the greater part of the tumour described in this paper, the stroma was quite sparse and limited to support for the myriad of blood vessels. But the most important features, of course, are the morphology and arrangement of the tumour cells. In the tumours described by Slye and Wells (1935) and Hansen (1949) and in the present tumour, the histological appearance could be summed up as exaggerated insular structure. In parts of case 2, one of the tumours described by Hansen (1949) and apparently the greater part of the first two tumours described by Cello and Kennedy (1957), the arrangement of the tumour cells gives the impression of small ducts or origin from already existing fine ducts. Similar arrangements corresponding to the "rosettes" in human tumours mentioned by Duff (1942) could be seen here and there in the tumour described in this paper. In normal pancreatic tissue, the cells making up such small ducts are considered totopotential, i.e. capable after appropriate stimulation of proliferating and differentiating into either acinar or islet cells. Their significance for the formal histogenesis of insulomata has been discussed by O'Leary and Womack (1934).

Cytoplasmic granules in canine tumour cells with tinctorial properties resembling those in normal islet cells have been demonstrated by Hansen (1949) and Cello and Kennedy (1957). O'Leary

TABLE 1

CANINE PANCREATIC INSULOMA

Summary of some major features

Report	*Breed*	*Age*	*Sex*	*Duration of clinical signs*	*Blood sugar levels, mg %*	*Site*	*Metastases*
Slye and Wells, 1935.. ..	Bull terrier	12½ yrs.	♀	2 years	30		Duodenal lymph node.
Hansen, 1949	(1) Boxer	8	♂	1 year	—		liver.
	(2) St. Bernard	8	♀	none (a)	—		none.
McGrath, (b) 1956	Mongrel	12	♂	6 months	37 (61 mg % 6 weeks after extirpation)	—	none.
Cello and Kennedy, 1957 ..	(1) Cocker sp.	6½	♂	6 months	35–50		portal lymph nodes, liver.
	(2) Boxer	5	♂	1 month	35		portal lymph nodes, liver.
	(3) "Red bone hound"	10	—	3 months	28*–56 *after 24-hours fast		portal lymph node.
Hansen and Krook, (c) 1958	(1) Fox terrier	9	♀	4 months	—		none.
	(2) Boxer	10	♂	1 month	—		none.
	(3) Dobberman pinscher	10	♂	several months	43–49		duodenal lymph node, liver.
	(4) Collie	7	♀	1 month	—		none.

FOOTNOTES TO TABLE 1

(a) Apparently non-functional. Pyometra. See page 453.

(b) Cites three other examples—two functional islet "adenomas", one "adenocarcinoma".

(c) Mentioned in a brief survey of active endocrine tumours.

and Womack (1934) have pointed out that some of the β-cells in both active and inactive tumours in human beings resemble normal β-cells very closely in their staining properties, but in many tumours the majority of the other cells diverge somewhat from the expected pattern when exposed to various fixatives and dyes. Hansen (1949) made a similar observation on one of his canine tumours.

There is one feature of the canine pancreatic insuloma described in this paper which is pertinent to a discussion of the periodicity of clinical signs. Over large portions of the tumour the specific cells had a heavily vacuolated but fat-free cytoplasm. This appearance closely resembles the hydropic degeneration of β-cells which can be produced by "overwork" (Allen, 1922) or by the injection of diabetogenic anterior pituitary extracts (Ham and Haist, 1941). Although this type of degeneration has not been recorded for other insulomas in human beings or dogs one wonders whether or not these particular degenerative changes in the tumour cells reflect cyclic activity of the tumour cells or at least a heavy strain on the antidiabetogenic-diabetogenic balance. Canine β-cells are apparently quite susceptible to hydropic degeneration; this change is much more common in spontaneous canine diabetes mellitus than in the human disease (Hjärre, 1927).

CONCLUSIONS

A non-metastasising pancreatic insuloma in an eight-year-old Boxer bitch is described. Cytoplasmic granules tinctorially resembling those of β-cells were demonstrated in some tumour cells; otherwise there was widespread and pronounced hydropic degeneration throughout the tumour. Although there was no opportunity to determine the blood glucose level in vivo, the anamnesis of recurring episodes of unconsciousness, a common sign of hypoglycaemia induced by release of insulin from these tumours, is taken as evidence that the tumour reported here was active.

Most of the 14 canine pancreatic insulomas which have been described previously were active; about half had metastasised when detected. Neither the duration of clinical signs nor blood glucose levels appear to be reliable guides for estimating the presence or absence of metastases and consequently the suitability of these tumours for surgical treatment.

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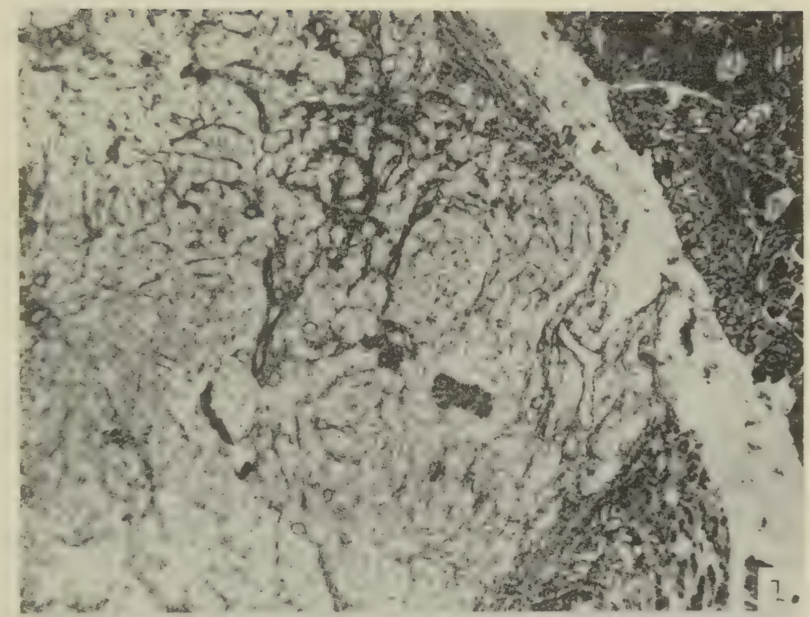
LEGENDS TO ILLUSTRATIONS

Fig. 1. Canine pancreatic insuloma (0·2158/59). A low-power view showing the tumour and adjacent pancreatic tissue. Note the thin capsule and the sparse, fine stroma. Gömöri's silver, × 50.

Fig. 2. Detail of same tumour. A cluster of cells with a fine granular cytoplasm surrounding a central space. The cytoplasm of the other tumour cells is heavily vacuolated. Inset—a typical small "rosette". H. & E., × 500.

Fig. 3. Specific staining for β-granules. The cytoplasmic granules in the central group of cells stain a deep blue. The other cells with a granular cytoplasm (periphery) do not show specific staining. The majority of the cells in this field, as is the case throughout the entire tumour, have a heavily vacuolated cytoplasm. Gömöri's chrome-haematoxylin-phloxine, × 500.



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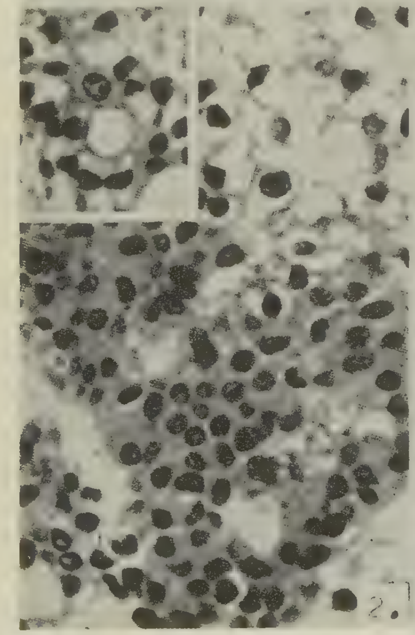

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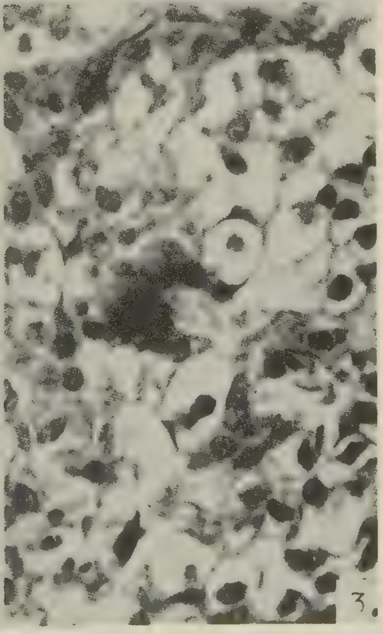

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STUDIES ON EPIZOOTIC LYMPHANGITIS IN THE SUDAN

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INTRODUCTION

Epizootic lymphangitis is a contagious disease of solipeds, caused by *Histoplasma farciminosum* (*Cryptococcus farciminosus*), and characterised by nodules and chronic suppurating ulcers on the skin, with inflammation of associated lymph vessels and nodes.

Henning (1956) reported that the disease exists enzootically in the Mediterranean countries, in Egypt and the Sudan, in India, China, Japan and several other parts of Asia. The first official record of the disease in Sudan was early in 1927 and in 1928 fifty-one cases were recorded.

Isolation of *H. farciminosum* has given difficulty and the earlier successful attempts were summarized by Bullen (1950, 1951) who used for primary cultures Hartley digest agar of pH 7·4 to which 10 per cent horse blood serum was added, and an incubation temperature of 37°C. On this medium colonies appeared as minute grey flakes between 12 days and 8 weeks. Ainsworth and Austwick (1958) reported that *H. farciminosum* is dimorphic, the parasitic phase is yeast-like and the saprophytic phase is mycelial being characterised by septate hyphae bearing thick-walled smooth chlamydospores. Bullen (1949) reported that it was possible to effect the mycelial yeast transformation in vitro by growing the fungus on blood agar at 37°C. in an atmosphere of 15 to 20 per cent CO_2.

At present the only sure diagnostic method depends upon microscopic appearance of the fungus. Bullen (1950) pointed out that it is not certain that the presence of the organism in typical superficial lesions is the only manifestation of the infection, but that it is possible that animals may receive mild transitory infection, or become carriers of the fungus without showing symptoms of the disease. Bennett (1931) described a primary interstitial pneumonia in mules and horses in the Sudan, which was not associated with skin lesions. In sections of the affected lung tissue an organism closely resembling *H. farciminosum* was seen, but as no cultural examinations were made its identity is in doubt (Ainsworth and Austwick, 1958). Hutyra, Marek and Manninger (1946) indicated a marked resemblance between epizootic lymphangitis on the one hand and sporotrichosis and streptotrichosis as well as some forms of cutaneous botryomycosis on the other. Smith and Jones (1957) state "the organisms of epizootic lymphangitis and histoplasmosis cannot be differentiated in tissue sections, hence cultures are necessary to distinguish between the two infections."

The reasons for the difficulty in the cultivation of *H. farciminosum* from infected tissue and the many failures of workers to isolate the organism are not yet understood. The main purpose of this paper is to describe methods of culture which permit the rapid luxuriant growth of the fungus from infected animal tissues.

METHODS

Material was collected from cases of epizootic lymphangitis in four horses, four mules and one donkey from the Blue Nile and Kordofan provinces.

A modified Littman medium (1955) was used comprising beef liver infusion, containing 5 g. thiotone, 500 ml.; beef lymph node infusion containing 5 g. thiotone, 500 ml.; beef spleen infusion, containing 5 g. thiotone, 500 ml.; dextrose, 10 g.; agar, 20 g.; streptomycin dihydrochloride, 50,000 μg.; penicillin (aqueous crystalline), 20,000 units; sterile sheep's blood, 100 ml.

500 ml. each of infusion of beef liver, lymph node and spleen, containing thiotone were mixed, and adjusted to pH 7·7, autoclaved at 15 lb. for 15 minutes, cooled and filtered through filter paper and the pH readjusted to 7·7. Ten grammes of dextrose were dissolved in this mixture, 20 grammes of agar were added and the mixture melted and autoclaved at 10 lb. for 10 minutes. The sterile melted agar base was then cooled to 50°C., and streptomycin and penicillin aqueous solution and 100 ml. sterile sheep's blood were added with sterile precautions. The solution was mixed and poured in sterile petri dishes and universal bottles, and then stored in the refrigerator after solidification.

Before inoculating the media the inoculum was treated with penicillin by adding 500 units per ml. of material and incubating at 37°C. for fifteen minutes. Primary cultures were made in capped universal bottles, four bottles being seeded from each case. At first duplicate sets of bottles were incubated, one set at 37°C. the other at 25 to 30°C.; incubation was later done only at 25 to 30°C.

RESULTS

Isolation of the Fungus

Attempts to isolate the fungus on a variety of media, including Sabouraud's and blood agar completely failed, due to contaminants (Staphylococcus, Streptococcus, Corynebacterium and *B. subtilis*). The first successful growth was obtained on the modified Littman's (1955) medium. Few bottles showed growth within 48 to 72 hours. Two types of colonies appeared, one small and moist which could be easily removed from the medium (this was found microscopically to be formed entirely of budding yeast-like cells) and the other a dry, white, larger colony firmly adherent to the medium, which on microscopic examination was found to be formed of septate mycelia. As soon as these colonies appeared a subculture on a plate was made from each type and incubated at 25 to 30°C. A pure growth of each type appeared within 48 hours.

The colony appeared circular, with radiating edges up to 1 cm. in diameter, raised from the surface, dome-shaped, differentiated into a greyish green centre and a light coloured periphery penetrating deeply into the medium with dark coloured reverse, firmly adherent to the medium, membranous and difficult to emulsify. Under the low power of the microscope the colony appeared to be formed of backed tufts of mycelia (Fig. 1), but under the high power it was found to be formed of septate mycelia, pseudomycelia and spores (Fig. 2).

The yeast-like colony appeared circular in shape, up to 2 mm. in diameter (Fig. 3), raised, with a smooth glistening surface and entire edge, in early growth whitish in colour, turning creamy on further incubation. Its consistency was soft and buttery, easy to remove from the medium and to emulsify. Under the low power of the microscope the colony appeared circular (Fig. 4), but under the high power it was found to be formed entirely of budding cells (Fig. 5), oval or circular, of different sizes averaging 3·5 × 4 μ. In general they were similar to those seen in infected tissues (Fig. 6). In fresh preparations examined by dark field illumination the cell-wall appeared double, containing finely granular cytoplasm and occasionally containing a vacuole. Budding usually took place from one end of the cell, and the daughter cell had a thinner wall.

Subculture on Liquid Media

The mould phase grew very easily in serum broth and in the BAI modification of Dorset medium (1934), incubated at 25 to 30°C. Within 72 hours growth appeared in the form of greyish green pellicles, leaving the column of fluid clear. Microscopic examination showed that the morphology of the organisms was exactly the same as on the solid medium.

In the yeast phase, scanty growth was shown on BAI medium at 25 to 30°C. Within 3 weeks slight turbidity and small flakes appeared floating on the surface, sedimenting quickly on shaking. There was a moderate powdery deposit. On microscopic examination the organisms were similar to those on solid medium except that they were more circular and larger in size (up to 7 × 7μ in diameter) and stained more easily with Gram stain. After 90 days incubation, subcultures on solid medium grew very luxuriantly within 72 hours incubation at 25 to 30°C.

Effect of Temperature

Duplicate sets of subculture from yeast-like colonies were prepared on liver-lymph-gland-spleen glucose blood agar media. One set was incubated at 37°C. and the other at a temperature varying from 25 to 30°C. Growth was much more luxuriant on plates incubated at 25 to 30°C. and when plates which were incubated at 37°C. for 5 days were removed for incubation at 25 to 30°C., the spread of growth and mass of culture were greatly enhanced.

When mould colonies were treated in the same way growth was found to be possible only at 25 to 30°C.

Effect of CO_2

Subcultures were made from the yeast-like colonies on liver-lymph-gland-spleen glucose blood agar on plates and divided into three sets: the first set was incubated at 37°C. in an atmosphere containing 15 to 30 per cent CO_2, the second at 25 to 30°C. in the same atmosphere and the third set at 25 to 30°C. without CO_2. Similar luxuriant growth was obtained in those plates incubated at 25 to 30°C. with or without CO_2, indicating that CO_2 is not essential for the growth of the yeast-like phase on that medium. Subcultures from mould colonies were treated in the same way and growth was obtained only on those plates incubated at 25 to 30°C. without CO_2.

Transition of Mould to the Yeast Phase

Liver-lymph-gland-spleen glucose blood agar proved to be an excellent medium for the production of the yeast phase of *H. farciminosum*. The mould phase grows well on the same medium at 25 to 30°C., but at 37°C. neither growth nor transition to yeast phase could be obtained. Exclusion of air from bottles of medium by a tight seal or the incubation of plates at concentrations of CO_2 varying from 15 to 30 per cent failed to effect the transition of mould to yeast phase. When mould colonies were grown on blood agar plates at 37°C. in an atmosphere of 20 per cent CO_2, yeast-like colonies appeared in sufficient numbers between the mycelial growth to permit their isolation in a pure state. In some instances a second transplant was necessary for the transition process.

Clinical Use

Out of 16 clinical specimens of epizootic lymphangitis grown on liver-lymph-gland-spleen glucose blood agar at 25 to 30°C., *H. farciminosum* was recovered in a pure state from 14 samples, indicating that this culture medium permits rapid growth and is highly useful in the isolation of the causative organism of the disease.

DISCUSSION

Epizootic lymphangitis, known locally as Saraga, is a fairly common disease among solipeds in the Sudan. In the last three years 138 cases were diagnosed in this laboratory, of which 72 were in horses, 49 in mules and 17 in donkeys, agreeing with the general conception that the disease is more prevalent among horses and mules than among donkeys. The highest incidence was recorded from the Blue Nile province with 63 cases, mainly in mules, and from Kordofan province with 53 cases. Despite the slaughter policy practised in the country, the disease is still prevalent with no great change appearing in the picture. In 1928, 51 cases were recorded and in 1959 43, with the highest incidence still in the same parts of the country. This state of affairs may be due to the presence of carriers which spread the disease without showing clinical symptoms. Bennett (1944) indicated that on account of the varied nature of Cryptococcus infection, many animals carrying the disease are liable to be missed.

The diagnosis of epizootic lymphangitis may be reached by the clinical symptoms and the finding of the fungus in infected material. In body tissues the organism occurs only as a double-contoured round or oval yeast-like cell reproducing by budding with only one bud being formed at a time. This description not only fits *H. farciminosum* but also other pathogenic fungi such as *H. capsulatum* (Smith and Jones, 1957). This fact has led some authors to doubt the work of Bennett (1931) on primary pneumonia in mules and horses due to *Cryptococcus farciminosus*. Although the usefulness of the microscopic examination of pus smears in the diagnosis of the disease cannot be doubted, nevertheless the final proof of infection should rest upon the isolation and identification of the specific aetiological agent, *H. farciminosum*. Recovery of this fungus from infected material has, in the past, presented considerable difficulties. In an endeavour to find a suitable medium for the isolation and identification of the organism it has been found that a liver-lymph-gland-spleen glucose blood agar medium is capable of effecting a satisfactory and rapid growth.

Salvin (1949) reported that *H. capsulatum* requires cysteine and cystine as well as biotin and reduced sulphur for the growth of the yeast phase. On the assumption that *H. farciminosum* would require the same nutrients, the medium under investigation was devised for the isolation of the organism. The addition of penicillin and streptomycin to the medium enhanced the isolation of the fungus by restricting bacterial growth. During the present study it was found that keeping the sample at room temperature and inoculating it without penicillin treatment lessens the chance of isolating the organism, but when the sample is refrigerated and penicillin added before inoculation or freshly seeded the chance of isolating the organism is greatly increased. Moreover antibiotic-resistant bacteria appearing in the sample exert an adverse effect on the recovery of *H. farciminosum*. This might be due to overcrowding, competition with fungus for food, and the more rapid growth of bacteria, or to the elaboration by bacteria of soluble substances toxic to the fungus. This suggestion is supported by the report of Littman (1955) that many antifungal substances derived from bacteria have been described, such as prodigiosin, violacein, pyocyanine, hemipyocyanine, circulin, polymyxin, bacillomycin, eumycin, fungistatin, mycosubtilin, and fluormycin, the last five being derived from *B. subtilis*, a common contaminant encountered during the present work. This might account for the failure of early workers as well as the difficulty experienced in isolating the fungus in the initial stages of this work. It was eventually decided that if fresh material was not available, samples should be collected in sterile containers, kept on ice and penicillin added to the inoculum to reduce bacterial growth and prevent the elaboration of antifungal substances which are active against *H. farciminosum*.

There has been considerable speculation about the factors which influence the phase of the fungus, i.e. as to when it forms mycelia and when it assumes the yeast-like form. Littman (1955), studying *H. capsulatum,* found that he could effect the transition of mould to yeast phase on liver-spleen glucose blood agar at 37°C., and following the advice of Salvin (1949) showed that the type of media and the temperature are the important factors in the transition. In the present study we obtained both phases on liver-lymph-gland-spleen glucose blood agar incubated at 25 to 30°C. and no transition of mould to yeast phase was obtained by incubation at 37°C. This was achieved by following the technique of Bullen (1949) of culturing the mycelial form on blood agar in an atmosphere of 15-20 per cent CO_2 at 37°C. Bullen was not able to use this method for the primary isolation of the yeast form of the fungus, however, and without CO_2 the culture could not be maintained on the yeast form. He concluded that the presence of CO_2 appears to play an essential part in the development of the yeast-like phase. On our medium the yeast phase could be isolated in primary cultures, and subcultures were maintained in sealed or unsealed bottles without any sign of reverting to mycelial type growth for over three months. On this medium CO_2 is not essential for the development or maintenance of the yeast-like phase of *H. farciminosum.*

Bullen (1951) reported that the optimum temperature for complete mycelial development of his culture was between 20 to 25°C. Growth was extremely slow, 2 to 3 months being required for maximum growth yield. On our medium maximum growth yield was obtained within 7 days at an optimum temperature between 25 to 30°C.

No effective control of epizootic lymphangitis can be achieved without a reliable diagnostic method. The development of the present culture medium not only makes it possible to explore the usefulness of an allergic or serological diagnostic method, but also the possibility of preparing a vaccine.

CONCLUSIONS

An agar culture medium containing infusions of beef liver, lymph gland, spleen, thiotone, dextrose, sheep blood, penicillin and streptomycin was prepared which supported a heavy growth of *H. farciminosum.*

Unenriched basal culture agars such as Sabouraud's and blood were unsuitable in recovering the fungus.

Luxuriant yeast phase growth of the fungus was readily produced and maintained on the medium for over 3 months.

Incubation of the plates between 25 and 30°C. was found to provide the best condition for the growth of the fungus.

An inhibitory effect of antibiotic-resistant bacteria was observed which explained the death of the organism in material stored at room temperature and justifies the need for collection of material in

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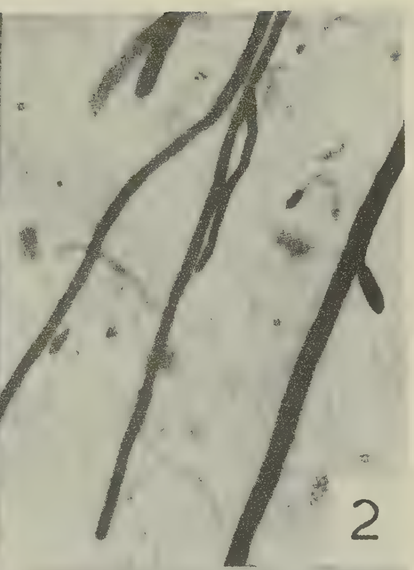

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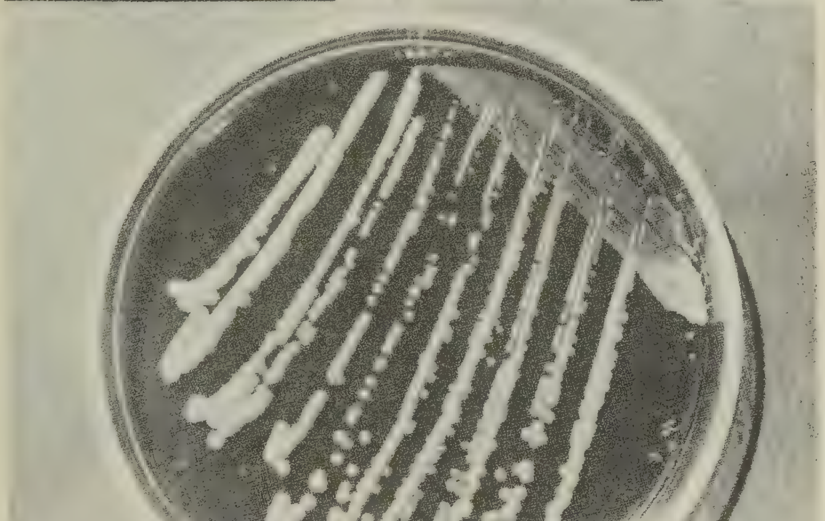

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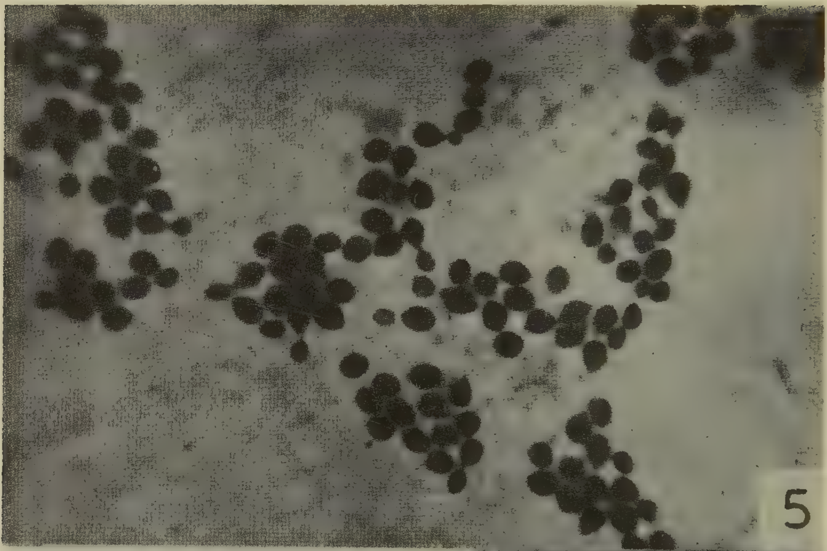

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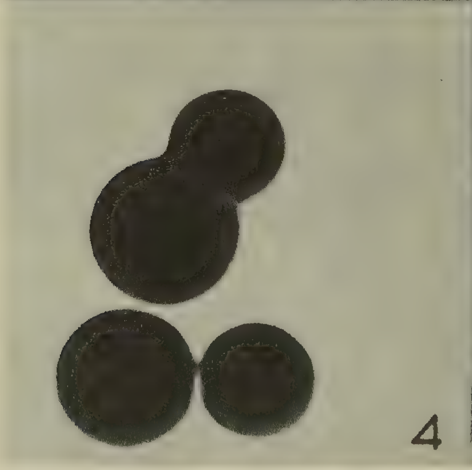

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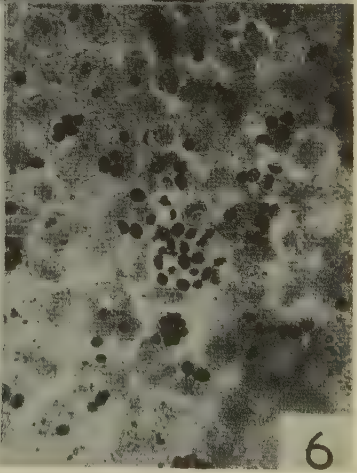

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Fig. 1. *H. farciminosum* : colony under low power.

Fig. 2. Same colony under high power showing septate mycelia, pseudomycelia and spores.

Fig. 3. Appearance of yeast-like colony.

Fig. 4. Yeast-like colony under low power. Colony appears circular.

Fig. 5. Yeast-like colony under high power. Colony shows oval or circular budding cells.

Fig. 6. Appearance of similar budding cells in infected tissue.

antibiotic solutions, refrigeration and prompt culturing as well as examination of fresh specimens.

The present investigation confirms the diaphasic nature of *H. farciminosum.*

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I wish to thank Dr. I. M. Khalil, Director, Ministry of Animal Resources, for permission to publish this work, Dr. A. A. Karib, Assistant Director of Research, for much help and advice, and El Tahir Eff. for his capable assistance in the preparation of the media.

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BERSAMA ABYSSINICA POISONING: THE CLINICAL AND PATHOLOGICAL PICTURE

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INTRODUCTION

Shrubs of the genus *Bersama* are common throughout Africa and the medicinal properties of the bark and seeds of some species have been reported by Watt and Breyer-Brandwijk (1932) and Dalziel (1937). The bark and seeds of the species *B. abyssinica* Fres. sub sp. *abyssinica* appear to have no medicinal uses, but the leaves have recently been incriminated in deaths of cattle in Uganda (Harker and Gourlay, 1959). This species occurs in Uganda, Kenya, Tanganyika, Ethiopia, Cameroons, Congo and possibly Northern Rhodesia (Eggeling and Dale, 1951; Verdcourt, 1950; Verdcourt, 1958).

The object of this paper is to describe the clinical and pathological picture seen in cattle naturally poisoned by the leaves of *B. abyssinica* sub sp. *abyssinica,* in experimental calves and rabbits following ingestion of leaf material from the shrub, and in mice following subcutaneous and intraperitoneal injection of a crude leaf extract.

MATERIALS AND METHODS

The first deaths occurred in cattle which were part of the milking herd belonging to the Livestock Experimental Station, Entebbe (LES). This herd consists of Nganda, Zebu and Kenana breeds of cattle. The calves experimentally fed with toxic material were from a herd of Zebu cattle belonging to the Animal Health Research Centre, Entebbe (AHRC). Rabbits and mice were from the small animal colony at the AHRC.

Leaves collected from shrubs of *B. abyssinica* on and within a radius of 4 miles of the LES, and fragments of leaves of *B. abyssinica* found in the rumen of the first cow that died were used for experimental purposes.

The rabbits were fed green leaves, either fresh or wilted for 6 hours, or were forcibly fed with air-dried leaf material roughly ground and made into a paste with water. The calves were drenched with dried leaf material which had been roughly ground and suspended in water.

Mice were inoculated subcutaneously, although a few were inoculated intraperitoneally, with an extract of leaf material prepared in the following manner: 2 g. dried leaves were ground finely with sterile sand in a mortar and taken up in 40 ml. of 50 per cent. acid alcohol. After standing for 10 minutes the mixture was filtered and the filtrate evaporated to dryness with very low heat under a fan. The residue was taken up in 5 ml. of 25 per cent acid alcohol and the resulting solution was inoculated into mice. Mice were also inoculated subcutaneously with an extract prepared from the leaf material obtained from the rumen of one of the dead cows. About 2 g. wet leaf material after standing in water for twenty-four days was filtered to remove the disintegrated leaf material, and the filtrate was evaporated until not quite dry. The residue was taken up in 4 ml. of 25 per cent. acid alcohol and centrifuged. The supernatant was shaken with chloroform, the chloroform was drawn off and evaporated, and the residue, taken up in 2 ml. of 25 per cent. acid alcohol, was used as the inoculum. The acid alcohol mixtures were prepared by adding 0·5 ml. of conc. HCl. to 100 ml. of 25 per cent or 50 per cent rectified spirit, respectively.

RESULTS

Clinical Picture

Natural

Cattle. During the night of the 4 to 5 June, 1959, the milking herd broke out of their paddock into an adjoining one. At 7.00 a.m. next morning, 5 animals were seen to be sick. A 4 years old Nganda cow of 733 lb. weight, which was severely affected, was dead by 8.30 a.m. Two more cows, one a 9 years old Nganda of 743 lb. and the other, a 5 years old Kenana of 632 lb., died at 11.30 a.m. and 4.30 p.m., respectively. The maximum intervals from possible time of eating the toxic leaves to the time of death were 12, 15 and 20 hours, respectively, for these 3 animals. The two remaining sick animals, both Nganda cows of 700 and 824 lb. weight respectively, recovered fully after 4 days illness. The two animals that did not die immediately and the two that recovered were treated with sodium thiosulphate, as arsenic poisoning was at first suspected. This treatment was discontinued soon afterwards when it became known that this poison was not involved.

The clinical symptoms shown by the 3 cows that died were similar, although one of them was in the terminal stages of toxicity when first seen. This animal was lying on its side with its limbs extended stiffly and its neck bent back. Its eyelids were blinking continuously and frequent violent tetanic convulsions occurred. The other 2 animals were also lying down but were able to rise and walk with difficulty. Their hind limbs appeared extremely weak and ataxic. One animal managed to stagger with assistance to a loose box but the other collapsed and had to be transported. Later their limbs became paralysed and they were no longer able to rise. They eventually became prostrate and, like the first animal, lay on their sides with their limbs extended and their necks bent back. The temperatures were sub-normal. Hyperaesthesia, nystagmus and trismus became marked and their eyelids blinked continuously. Violent tetanic convulsions occurred before death. The two LES cows that recovered from the effects of the toxin showed signs of muscular weakness, lethargy and inappetance. On the second day one was scouring and appeared to be in pain. They ate a little concentrate but refused grass, and still showed some signs of weakness. By the fourth day they had both recovered, although their milk yields and appetites were reduced.

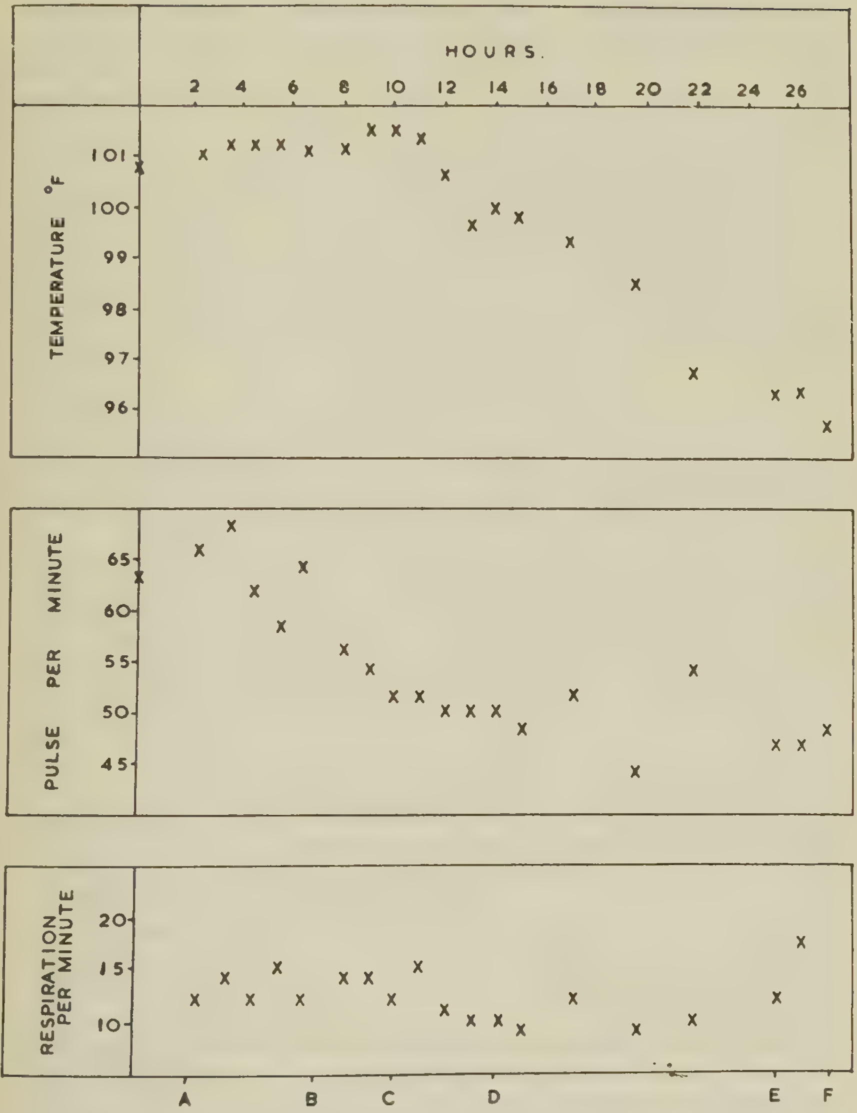
Experimental

Cattle. On 16 June, 1959, a 5½ month old male calf of 82 lb. weight was drenched with 4 g. of dried leaf material and was destroyed in extremis 25 hours later. On 7 and 9 December, 1959, two male calves 10 months of age and 220 and 214 lb. weight, respectively, were each drenched with 14 g. of dried leaf material. They both recovered fully after 2 and 3 days illness respectively.

The initial symptom seen in the first experimental calf was an apparent weakness of the hind limbs indicated by a swaying of the posterior part of the body and the animal standing with its fore-legs well apart. The hind limb weakness progressed until any attempt to walk caused the animal to stagger and almost fall. Later the fore-limbs became affected and the animal was unable to stand. It lay in an upright position with its limbs drawn up under it and the head either extended forward with the chin resting on the ground or twisted back on its flank. Slight tremors of the head and ears were seen, and the extremities were cold. The animal later lay on its side with its limbs extended stiffly and its neck arched back. Although the limbs were extended they would not support the weight of the animal if it was placed on its feet. If the animal was moved it momentarily drew up its legs to its body. The pupils were contracted and did not respond to light, the pulse was weak although the heart was still beating strongly and the respirations were shallow with a "double lift" action. The animal appeared very alert, its ears twitching continuously, and it did not appear to be in pain at this stage. The anus protruded as though due to lack of muscle tone, and urine was passed in dribbles. Following the relatively quiescent symptoms the next phase was characterised by progressive hyperaesthesia terminating in violent tetanic convulsions. The breathing became very irregular and shallow, the pulse very weak and the temperature continued to fall. Lateral nystagmus and trismus were seen and the eyelids blinked continuously. The hyperaesthesia became progressively more severe frequently requiring no stimulus to initiate a convulsion. The pupils became dilated and did not react to light change. Between convulsions the animal appeared alert and fully conscious. It became gradually weaker and the convulsions more frequent and it was eventually destroyed in extremis. Details of the temperature, pulse and respirations of this calf are given in Fig. 1.

One of the experimentally fed calves that recovered showed only a very slight transient unsteadyness and fine tremors of the head, ears and limb muscles. The other calf showed considerably more severe symptoms. Within 4 hours of drenching it was salivating and was unsteady on its limbs. During the next 24 hours its condition altered little, but the salivation ceased, it appeared tranquilized, preferred to lie down and had difficulty in rising when disturbed. Once on its feet it had great difficulty in standing and would prop

Fig. 1



Temperature, pulse and respiration of calf 1 experimentally fed with leaves of *Bersama abyssinica*. A—time of drenching with leaf material. B—first signs of hind limb weakness. C—calf unable to stand. D—lying outstretched on its side. E—convulsions, trismus and nystagumus. F—destroyed in extremis, respirations too shallow for accurate counting.

its hind quarters in a corner of the loose box. Any attempt to walk would cause it to stagger and fall. There was twitching of the ears, slight tremors of the head and hyperaesthesia of the skin. The temperature was subnormal, but the pulse and respirations did not alter appreciably. The symptoms gradually diminished over the next 24 hours and the temperature slowly returned to normal. The animal was still unsteady on its legs but remained standing with back arched and head held low. By the fourth day after drenching the animal appeared normal.

Rabbits. Eight rabbits were fed with toxic material; 4 died and 4 recovered after showing symptoms of poisoning. Rabbits 1, 4 and 6 were given fresh or slightly wilted leaves to eat. The remaining five had to be forcibly fed with ground dried leaves mixed into a paste with water because they refused to eat the fresh leaves. All except the eighth were starved for 24 hours prior to receiving the toxic material. Details of the rabbits and material fed are given in Table 1.

TABLE 1

DETAILS OF RABBITS FED WITH LEAVES OF *BERSAMA ABYSSINICA*

No.	*Approx. weight (Kg.)*	*Date fed*	*Method*	*Approx. dry wt. ingested (g.)*	*Result*
1	2	June 1959	Eaten	1·0	Died within 6 hours
2	2	,, ,,	,,	0·25	Died within 10 hours
3	2	,, ,,	,,	0·1	Recovered
4	1·5	Oct. 1959	,,	1·5	,,
5	2	,, ,,	Forcibly fed	2·0	,,
6	2	Nov. 1959	Eaten	0·2	,,
7	2	,, ,,	Forcibly fed	1·25	Died within 19 hours
8*	2	Dec. 1959	,, ,,	1·5	Died within 44 hours

*Not starved before experiment.

The clinical symptoms exhibited by the rabbits that died were as follows: 1½ to 3 hours after receiving the leaf material there were signs of incoordination and photophobia. Later the animals assumed a hunched-up position and became very sleepy, closing their eyes and breathing deeply and slowly. On being disturbed they would wake with a start but would quickly fall asleep again. Frequently the anterior half of the body would keel over on its side while the posterior part would remain upright. Later twitching of the eyelids and champing of the jaws became noticeable. The respirations became very rapid and hyperaesthesia and nystagmus were evident. The ears and extremities became very cold and the temperature subnormal. Eventually, any loud noise such as clapping of the hands would initiate severe opisthotonic convulsions. Between convulsions the animals appeared completely relaxed and the respiration and pulse rate were slow. Following each convulsion, however, the pulse became very rapid and irregular and the respirations very fast and shallow, frequently having a "double lift" action. Towards the terminal stages the convulsions became more severe and frequently required no external stimulus to initiate them. The eyelids blinked continuously and the animals gripped the cage wires tightly in their teeth. Nystagmus, lacrimation and salivation were marked. The pupil reflex became very sluggish. During these later stages the rabbits occasionally appeared to be in severe pain since, following a convulsion, they would emit a prolonged high pitched scream. Defaecation frequently occurred during a convulsion and the droppings were usually covered with mucus.

The clinical symptoms shown by the rabbits that recovered from the effects of the toxin first became evident about 3 hours after feeding. They were incoordination, limb weakness, sleepiness, hyperaesthesia, rapid respirations and subnormal temperature. Rabbit 4 was the most severely affected of these and in addition to the above symptoms showed nystagmus and suffered convulsions. Rabbit 3 showed a marked forelimb weakness or paralysis which remained for a considerable time. Recovery in all cases was slow, and the incoordination and paralysis gradually disappeared over a period of up to 5 weeks. During that time the animals continued to eat spasmodically, mild chorea frequently developed and the vestibular apparatus invariably appeared affected as their sense of balance was poor.

Mice. Early in the investigation a test using a crude extract of dried leaves injected subcutaneously and intraperitoneally into mice was used as a means of determining the toxicity of various batches of leaves from different Bersama plants. Doses of 0·1 and 0·2 ml. of the leaf extract were the standard inocula.

The clinical symptoms seen in the mice were very consistent. In the majority of cases where leaf extracts of average toxicity were injected the first symptoms appeared ½ to 3 hours after subcutaneous inoculation and 10 to 30 minutes after intraperitoneal inoculation. The mice first became hyperexcitable and very active, and frequently appeared fierce and aggressive if disturbed. Later they appeared to develop photophobia, many of them hiding their heads in corners. Soon after this phase the mice assumed an unusual posture, the shoulders were hunched up and the lumbar region was held low, the spinal column appearing to bend down in the middle. This was followed by progressive weakness of the limbs until they were no longer able to support the weight of the body. The mice collapsed on their abdomens with their hind limbs under them and their fore limbs spread out sideways and progressed by paddling movements. Eventually they were no longer able to move and lay prostrate, limbs splayed out and breathing very rapidly. Finally the mice fell on their sides. Hyperaesthesia gradually increased until even the slightest physical or sonic stimulus would send them into opisthotonic convulsions. Later the convulsions occurred without any detectable stimulus. The animals became cyanosed and their eyes frequently protuded markedly. Death invariably occurred following an opisthotonic convulsion, when the mice would assume a characteristic posture with their necks arched, muzzles drawn down onto their chests, hind limbs and tails fully extended and forelimbs clasped across their chests; they would die in this position. Frequently after death the pupils would fully constrict.

Mice receiving a sublethal dose of toxic material exhibited similar symptoms to those noted above. Weakness of the limbs and the unusual hunched posture always occurred. Occasionally mice suffered convulsions and still recovered. Recovery was usually complete within four days.

Pathological Picture

Cattle. Post-mortem examinations were performed on the 3 cows that died and on the experimental calf that was destroyed.

Externally the carcasses appeared normal with no signs of scouring or salivation. In all cases the conjunctivae were congested and the mucous membrane of the buccal cavity and the tongue was slightly cyanosed. Internally, the heart and lungs were normal, but the upper respiratory tract was cyanosed. The liver and kidneys showed slight congestion in all cases. The spleen was normal except in one case where a subcapsular haemorrhage was present. The rumen, reticulum and omasum were normal. The abomasum showed signs of slight inflammation in all cases. In one case, however, this could be attributed to *Haemonchus contortus* which was present in quite large numbers. The intestines were normal or slightly congested. The bladder was usually distended with urine, and in one case the mucous lining was inflamed.

Histological preparations of the liver, kidney and myocardium were examined from two of the cows and from the experimental calf. In addition preparations of the spleen, brain and the optic and sciatic nerves of the latter were also examined. The liver showed minimal focal degeneration in the first cow that died, slightly more severe centrolobular degeneration in the second cow and a focal haemorrhagic necrosis in the experimental calf. In all cases the kidneys showed no abnormalities except in the first case where lipofuscin was present in the tubular cells. The spleen in the experimental animal showed focal necrosis and follicle hyperplasia. No abnormalities were seen in the other tissues and the nerves showed no evidence of demyelination.

Rabbits. Post-mortem examinations were performed on the carcasses of all the rabbits that died except the first. The liver in all cases was pale, but paler mottled areas occurred all over the surface.

The spleen and kidneys were also pale. The heart was contracted in systole. In rabbit 8 the stomach was full of very dry mash in which Bersama leaf material could be seen. The small intestines were empty except for a pale green mucus.

Histological preparations of the liver, spleen, kidney and myocardium from rabbits 7 and 8 and, in addition, the brain and spinal cord of rabbit 7, were examined. The liver showed a widespread centrolobular cytoplasmic necrosis with the nuclei apparently normal in rabbit 7, and a widespread centrolobular degeneration with focal areas of necrosis in rabbit 8. The kidneys showed early tubular degenerative changes in rabbit 7 and tubular necrosis in rabbit 8. All other tissues examined appeared normal.

Mice. Post-mortem examinations of 7 of the mice that died following injection of toxic extracts consistently showed a pale pink spleen and usually a pale mottled liver. The kidneys were pale with small haemorrhages occasionally present on their surface. The heart was generally contracted in systole. The bladder was invariably distended with urine and the penis frequently erect. The alimentary tract was normal. In 11 mice that had recovered from a sublethal dose of extract and were subsequently killed, the spleen appeared quite normal.

Histological preparations of liver, spleen, kidney, myocardium and brain were examined. In acute cases the liver showed a slight centrolobular degeneration and in less acute cases a diffuse necrosis. A generalised cytoplasmic dissolution of the liver in which the nuclei were apparently normal was seen in 2 instances where the mice had apparently recovered from the effects of the toxin and were destroyed 48 hours later. In cases where the mice had been destroyed 4 days or more after recovery the liver appeared quite normal. All other tissues examined appeared normal.

DISCUSSION

The incrimination of *Bersama abyssinica* sub sp. *abyssinica* as the cause of cattle deaths on the LES Entebbe was based on strong circumstantial evidence. The history was very suggestive of poisoning and pathological, bacteriological, virological and protozoological examination of materials from the carcasses failed to show any other cause of death: moreover, chemical examination of rumen contents, liver and blood, failed to demonstrate the presence of arsenic or hydrogen cyanide, both common forms of poisoning in Uganda. Physical examination of the rumen contents showed a mainly graminaceous diet, but also showed that the 3 cattle had eaten a few leaves of one particular dicotyledonous plant.

From the small pieces of leaf material it was possible partially to reconstruct a leaf, and a search was made in the paddock concerned for the parent plant. A shrub was eventually found having leaflets similar to the reconstructed leaf from the rumen. This shrub was later identified as *B. abyssinica* Fres sub sp. *abyssinica*. Leaves of this shrub were not then known to be poisonous but they were fed to a rabbit with rapidly fatal results.

Subsequent investigations produced further evidence that the leaves of this shrub were responsible for the cattle deaths. First, a calf fed with leaf material died, showing symptoms and a pathological picture similar to the naturally affected animals. Secondly, an extract of the leaf material from the rumen of the dead animal gave similar symptoms to extracts of leaf material from the shrub when injected into mice. It appears, therefore, that there can be no doubt that this plant was responsible for the deaths of the LES cattle. Apart from the circumstantial evidence regarding the 3 LES cattle, it has been shown experimentally that leaves from this shrub are toxic to rabbits and calves following ingestion and mice following injection of an extract, and it is probable that they are also toxic to other livestock and man.

Differences noticed between June and December in the amount of leaf material necessary to cause toxic symptoms or death in experimental animals, possibly indicates a seasonal variation in toxicity of the leaves. Rabbits also appeared to find the leaves less palatable in the later months of the year.

The clinical and pathological picture in cattle, rabbits and mice was essentially the same, except that rabbits became excessively sleepy in the early stages. This was not noticed in the other animals although one of the experimental calves, which, before the experiment and again after recovery was extremely wild and unmanageable, became very quiet and tractable after being fed toxic material even though the toxic effects were not severe. The convulsions differed slightly in the different species. In rabbits and mice the spasms were clonic whereas in cattle they were tonic, the animals being in a stage of perpetual opisthotonus with intermittent violent spinal convulsions. Respirations were irregular and shallow in cattle but in rabbits and mice they were extremely rapid in the later stages.

The poisonous principle still remains to be isolated and its nature determined. From the clinical and pathological findings, however, it does not appear to be a well known alkaloid or glucoside. Its main effect appears to be stimulation of the nervous system, particularly the spinal cord (in much the same way as strychnine), although the initial symptoms appear to be depressive in character, particularly in rabbits. It may be that more than one toxic principle is involved. The liver damage noted in mice may have been caused partly by tannins (Armstrong, Clarke and Cotchin, 1957) removed from the leaves by the crude method of extraction used, as the toxic material was administered parenterally. In the other animals, however, it would appear that the liver damage was not due to the effects of the tannins as the leaf material was ingested and insufficient tannin would have been absorbed to give rise to liver damage (Handler and Baker, 1944). Rabbit 8 survived an unusually long time after administration of the toxic material and it is of interest that this rabbit was not starved before use and on autopsy the stomach was seen to be very full of dry mash in which Bersama material could be seen.

The incidence of poisoning in Uganda caused by this shrub is unknown but it may be common as the shrub is widespread throughout the Protectorate. The clinical symptoms alone are not sufficiently characteristic to be diagnostic and under African field conditions may be confused with tetanus, botulism, heartwater, ephemeral fever, turning sickness, peracute nagana, strychnine poisoning and possibly other conditions. Diagnosis at present is dependent on clinical history, clinical symptoms and demonstration of leaf material in the rumen at autopsy. Identification of leaf material is difficult as the leaves of the shrub have no outstanding physical features. If, however, sufficient of the leaf material could be obtained from the rumen in suspected cases of poisoning, an extract could be prepared for injection into mice.

CONCLUSIONS

The leaves of the shrub *Bersama abyssinica* Fres sub sp. *abyssinica* have been incriminated in the deaths of 3 cows in Uganda. Leaves from this shrub were shown experimentally to be toxic to calves and rabbits following ingestion and to mice following subcutaneous and intraperitoneal injection of the leaf extract.

The clinical and pathological picture in cattle, rabbits and mice is essentially the same, comprising limb weakness leading to paralysis, hyperaesthesia, subnormal temperature, weak irregular pulse, irregular shallow respirations, trismus, nystagmus, opisthotonus and spinal convulsions leading to death, generally within 24 hours. In rabbits in the early stages there is a period of intense sleepiness. The pathological picture consists of degenerative changes in the liver and kidneys.

It is suggested that the toxin is not a well known alkaloid or glucoside and that its primary action is on the spinal cord.

Diagnosis of poisoning due to this shrub is at present dependent on clinical history, clinical symptoms and the demonstration of toxic leaf material in the rumen contents.

ACKNOWLEDGMENTS

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EXPERIMENTS ON THE TRANSMISSION OF CATTLE LUNGWORM INFECTION

By

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INTRODUCTION

Observations on the bionomics of the free-living larvae of the cattle lungworm, *Dictyocaulus viviparus,* showed that the proportion of non-infective larvae reaching the infective stage was largely determined by climatic factors which influenced both the rate of development of the larvae and their survival (Rose, 1956). Other experiments (Michel and Rose, 1954; Rose and Michel, 1957) demonstrated that the success of the infective larvae in reaching herbage depended more upon the extent to which the faeces and the larvae in them were spread, than on the surprisingly limited migratory activities of the larvae. This seemed to suggest that the ingestion of appreciable numbers of infective larvae could take place only if herbage contaminated with faeces was ingested. Observations on the grazing behaviour of cattle in relation to bovine faeces (Michel, Ollerenshaw and Rose, 1956) suggested that lightly contaminated herbage was, in fact, ingested while obvious deposits were avoided.

These experiments, while they elucidated some of the factors involved in the transmission of infection, could give no idea of their relative importance. Accordingly a series of experiments was set up with a view to determining the relative importance of climatic conditions, the distribution of faeces over the herbage, and the grazing behaviour of the bovine, in relation to the transmission of lungworm infection.

METHODS

Small paddocks were contaminated with infected faeces in various ways and at different times of the year. These paddocks were then grazed by susceptible calves. The numbers of worms which they picked up were determined, and used as an index of the extent to which the transmission of infection had taken place. Four repetitions of the experiments were carried out, starting on 10th August, 1956 (Summer), 26th October (Autumn), 30th January, 1957 (Winter), and 1st May (Spring), respectively. The paddocks, each ⅛th of an acre in area, were laid out on a three year ley consisting mainly of cocksfoot and clover. They were grazed with sheep prior to the start of each experiment in order that the sward should be of comparable length. Clean paddocks were used for each repetition.

Infected faeces were collected from a number of stalled calves and mixed thoroughly to ensure an even distribution of larvae. A constant amount of 82½ lb. of faeces, containing approximately half a million non-infective larvae, was used to contaminate each paddock. The faeces were of three different consistencies designated "solid", "semi-fluid" and "fluid". The "solid" faeces were as passed by stalled calves. The "semi-fluid" and "fluid" faeces were prepared by the addition of 200 ml. and 450 ml. of water, respectively, to each 1 lb. of solid faeces. Four paddocks, A, B, C and D, were contaminated with the faeces. The "fluid" faeces were widely distributed over paddock "A" to cover as great an area as possible. The "semi-fluid" faeces were evenly distributed over paddock "B" in the form of small pats. The "solid" faeces were evenly distributed over paddocks "C" and "D" as large pats each weighing 1½ lb. Sheep were then introduced into paddock "D" so that the pats were well trampled. This resulted in a scattering of some of the pats, but the faeces were not as thoroughly dispersed as in paddocks "A" and "B".

Two Jersey calves, between two and three months old, and reared lungworm-free were turned on to each of the paddocks. In order that they should have the same length of time in which to pick up infective larvae, the rate of development of larvae in faeces kept outside the paddocks was observed, and after the larvae had reached the infective stage the calves were left on the paddocks for a further three weeks only. In this way reinfection of the paddocks was precluded as the parasitic phase of the lungworm life cycle takes at least twenty-one days for its completion. The calves were autopsied immediately after removal from the paddocks and their lungs examined for worms using the method described by Michel (1954).

During the course of the experiments the grazing behaviour of the calves was observed and after their removal from the paddocks the pasture was examined to determine to what extent the faecal pats originally placed there had been dispersed.

The rainfall and minimum and maximum temperatures were recorded daily.

RESULTS

By carrying out four repetitions of the experiments, one in each season, it was possible to compare the transmission of infection under four different sets of climatic conditions. It is evident from the total numbers of worms picked up by the calves at the different seasons that climate had a very considerable effect on transmission (Table 1). The seasonal differences in the extent of transmission can be related to the effects of climate on the rate of development and the survival of the larvae, which also varied with the season (Table 2). The rate of development is known to be influenced by temperature. As the temperature falls so the rate of development slows down (Rose 1956). The seasonal variations in rates of development (Table 2) can, therefore, be attributed to seasonal differences in temperature (Fig. 1).

These variations in the rate of development have a bearing on the numbers of larvae which became infective at different times of the year. Many larvae die during the first three weeks of their free-living existence (Michel and Rose, 1954), so that when the rate of development was slow in the autumn, and even slower in the winter, many larvae died while still non-infective, whereas in the spring and summer, when development was rapid the larvae were non-infective for only a very short time and the majority succeeded in becoming infective (Table 2). Temperature also influences survival. The non-infective larvae are rapidly killed by freezing (Rose, 1956). This would further reduce the numbers which succeeded in becoming infective in the autumn and winter, when the temperature was frequently below freezing point (Fig. 1). Thus, as a result of the seasonal variations in climatic conditions, there were appreciable differences in the numbers of larvae which succeeded in becoming infective in the four repetitions which, in turn, influenced the extent to which transmission took place (Table 2).

TABLE 1

NUMBER OF WORMS RECOVERED FROM EACH PAIR OF CALVES

Paddock	*Faeces*	*Worms picked up during*			
		Summer Aug. 10th—Sept. 3rd	*Autumn Oct. 26th—Nov. 29th*	*Winter Jan. 30th—Mar. 10th*	*Spring May 1st—May 25th*
A.	Fluid	1,236	42	43	1,392
B.	Semi-fluid	64	2,768	23	5,581
C.	Pats	221	980	29	3,550
D.	Pats trampled by sheep	232	1,500	92	1,749
	Total	1,753	5,290	187	12,272

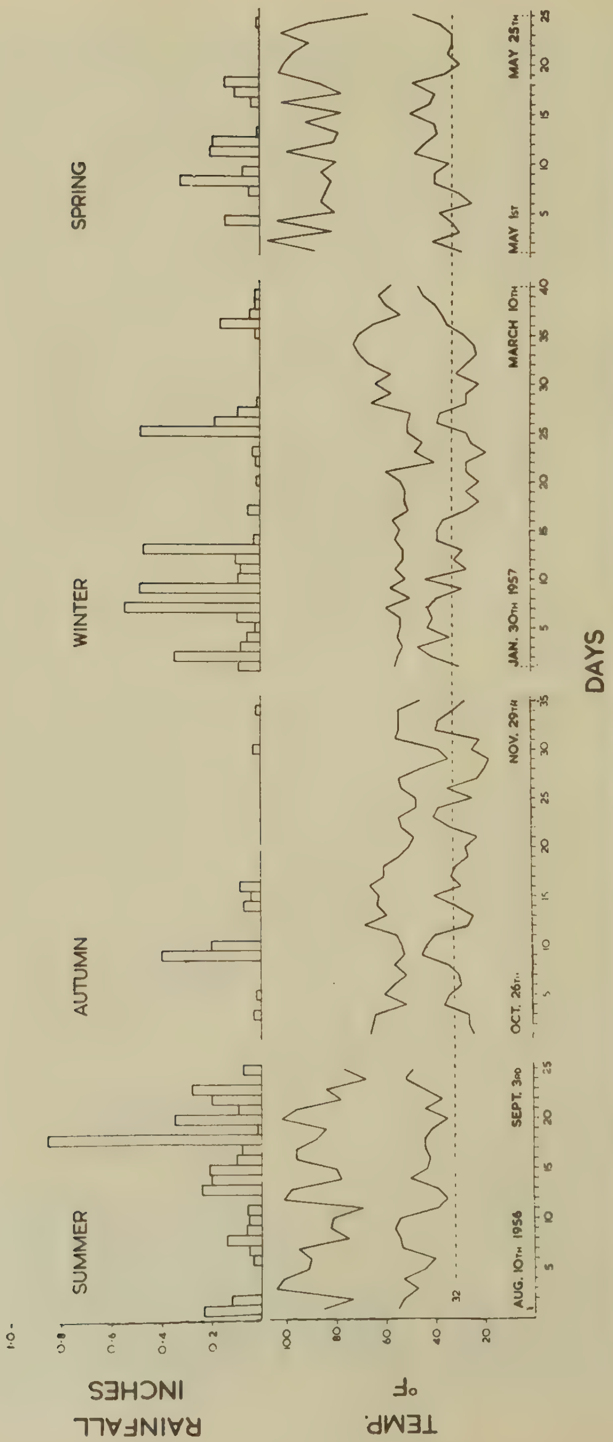
TABLE 2

RATE OF DEVELOPMENT AND SURVIVAL OF LARVAE IN FAECAL PATS KEPT OUTSIDE THE PADDOCKS

	Summer	*Autumn*	*Winter*	*Spring*
No. of days for development of larvae to infective stage	3–4	11–14	16–19	3–4
Approximate percentage mortality of non-infective larvae	5%	50%	95%	5%
Approximate percentage of larvae reaching the infective stage . .	95%	50%	5%	95%

The seasonal variations in transmission cannot be explained solely on the basis of mortality of the non-infective larvae, however, as equal numbers of larvae in faeces became infective, in both spring and summer (Table 2) although fewer worms were picked up in summer than in spring (Table 1). During the summer, transmission

Fig. 1



Meteorological data. Daily minimum and maximum ground temperature—°F, and daily rainfall in inches during each experiment.

can be limited by the rapid mortality of infective larvae as the result of hot and dry climatic conditions (Rose, 1956; Rose and Michel, 1957). Such conditions did not, however, prevail during the present observations. Temperatures were not appreciably higher in summer than in spring, and rainfall was both heavy and frequent (Fig. 1). As a result of this heavy rainfall, the infective faeces disappeared rapidly in summer, being washed into the soil (Table 3). As the lungworm larvae assume an inactive coiled form after becoming infective, and do not migrate from the faeces to any great extent (Michel and Rose, 1954), it is likely that they too were washed off the herbage into the soil. In spring, when there was less rain (Fig. 1), the faeces persisted longer (Table 3), and were able to serve as reservoirs of infection, in the manner described by Rose and Michel (1957), from which infective larvae could be transferred to the herbage throughout the grazing period. Although comparable numbers of larvae became infective in both spring and summer, it appears that they remained available to the calves for a shorter time in the summer with the result that fewer were picked up than in the spring. The amount of rainfall is thus another climatic factor which can influence the extent to which the transmission of infection takes place.

TABLE 3

NUMBER OF PATS OF INFECTIVE FAECES RECOGNISABLE AFTER REMOVAL OF THE CALVES FROM THE PADDOCKS

Paddock	*Faeces*	*Recognisable faecal pats*			
		Summer	*Autumn*	*Winter*	*Spring*
A.	Fluid	Not measurable			
B.	Semi-fluid	0	9	0	21
C.	Pats	2	26	8	42
D.	Pats trampled by sheep	4	17	6	23

The way in which the faeces were distributed over the paddocks did not have any appreciable effect on the transmission of infection during the winter. Relatively few worms were picked up by the calves on all the paddocks. The heavy mortality of the non-infective larvae appeared to be the most important factor affecting transmission at this time. In the spring, summer and autumn, however, there were appreciable differences in the numbers of worms picked up by the calves on the different paddocks (Table 1). This would seem to suggest that the transmission of infection had been influenced by the way in which the faeces were distributed. There was not, however, any consistent relationship between the initial manual distribution of the faeces and transmission. The number of worms picked up by the calves was not directly related to the area over which the faeces had been spread initially, as might have been expected. In order to understand this it is necessary to consider the process of distribution as a whole together with the grazing behaviour of the bovine.

The initial manual distribution of the faeces was only a transitory stage in a continuous process. The faeces were distributed throughout the course of the experiments by such factors as rainfall and the calves themselves. The extent to which this took place varied according to the season so that although the initial manual distribution was the same on comparable paddocks, this was not true of the complete process (Table 3).

TABLE 4

OBSERVATIONS ON THE GRAZING BEHAVIOUR OF THE CALVES

Paddock and faeces	*Summer*	*Autumn*	*Winter*	*Spring*
A. Fluid	Perimeter only grazed at first then paddock evenly grazed	Perimeter only grazed at first then paddock unevenly grazed	Evenly grazed	Perimeter only grazed at first then paddock unevenly grazed
B. Semi-fluid	Tussocks around pats avoided, remainder evenly grazed	Tussocks around pats avoided, remainder evenly grazed	Tussocks around pats avoided, remainder evenly grazed	Tussocks around pats avoided, remainder evenly grazed
C. Pats	,, ,,	,, ,,	,, ,,	,, ,,
D. Pats trampled by sheep	,, ,,	,, ,,	,, ,,	,, ,,

The distribution of the faeces can assist the infective larvae to reach the herbage, but in order that they can be effective in the transmission of infection, they must be ingested by the grazing bovine. The grazing behaviour of the bovine must, therefore, have a bearing on the transmission of infection. In the present experiments, the calves did not follow the same pattern of grazing on all of the paddocks. Those on the 'B', 'C' and 'D' paddocks grazed in a similar manner throughout, but this was not true of the calves on the 'A' paddocks (Table 4). In the spring and autumn, these calves appeared to be avoiding the area over which the faeces and larvae had been distributed. They did, in fact, pick up appreciably fewer worms than did the calves on the other paddocks (Table 1). In the summer, the contaminated area was again avoided for the first few days, but subsequently the whole paddock was evenly grazed and the calves on the 'A' paddock became the most heavily infected (Table 1). It is not altogether clear why there should have been these differences in the grazing behaviour of the calves on the 'A' paddocks. The most likely explanation is that in the spring and autumn, when the faeces disappeared slowly, the contaminated herbage remained unpalatable for a longer period of time than in the summer, when the faeces disappeared more rapidly (Table 3).

It would seem, therefore, that the effects of the distribution of faeces and grazing behaviour of the bovine on the transmission of infection must be considered together. The widespread distribution of faeces will help the infective larvae to reach the herbage, (Rose and Michel, 1957), but the transmission of infection will be enhanced only so long as contamination of the herbage is not so great as to render it unpalatable to the bovine.

DISCUSSION

In considering the relative importance of climatic factors, distribution of faeces and grazing behaviour of the bovine in relation to the transmission of lungworm infection, climatic factors are the most important of the three by virtue of their direct influence on the rate of development and survival of the lungworm larvae. When climatic conditions are such that only a small proportion of larvae become infective, as in the winter, transmission is negligible, irrespective of the distribution of faeces and grazing behaviour. In contrast, when climatic conditions are conducive to the development and survival of the larvae, the distribution of faeces, which itself is influenced by climatic factors, and the grazing behaviour both play a considerable part in limiting or facilitating transmission.

CONCLUSIONS

Experiments were carried out to determine the relative importance of climatic conditions, the distribution of faeces over the herbage and the grazing behaviour of the bovine animal in relation to the transmission of lungworm infection.

Climatic conditions were considered the most important, but under certain conditions the other factors had an appreciable effect on transmission.

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I wish to thank Mr. J. F. Michel for his interest and advice and for supplying the infected faeces. I also wish to thank Dr. S. B. Kendall for his critical reading of the original manuscript.

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THE COMPLEMENT FIXING ANTIBODY RESPONSE OF CALVES TO *DICTYOCAULUS VIVIPARUS*

I. Exposure to Natural and Experimental Infection

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INTRODUCTION

The pattern of the complement-fixing antibody response to experimental infection and re-infection with *Dictyocaulus viviparus* in calves has been reported by Weber (1958) in America and by Jarrett, Jennings, McIntyre, Mulligan, Thomas and Urquhart (1959) in this country.

The former used an adult whole-worm antigen prepared by a low-temperature method and a complement fixation test of the serum dilution type, employing the end-point of complete inhibition of haemolysis and 2 full units of complement. The initial antibody response was detected 12 days after infection and the titre rapidly reached a peak 4 days later. The titre then remained fairly constant (the plateau period) until the 63rd day. This type of response occurred in calves initially exposed and in those re-exposed on one or more occasions. Antibody levels after the 63 day period showed some variation according to the type of treatment. Calves initially infected and those re-exposed once, showed a decline in titre over the succeeding months except for one calf whose titre remained high for one year after a single infection. The titres of several animals receiving multiple exposures remained high for several months in the absence of further infection.

Jarrett *et al.* (1959) used a heated adult whole-worm antigen prepared by the method of Stewart (1950) and the serological technique employed was a quantitative complement fixation test of the 50 per cent haemolysis unit type. They showed that with initial infections, whether as a single dose of 2,000 larvae or 25 doses of 300 larvae at intervals of 2 to 3 days, the antibody response was first detected at 30 to 35 days and reached a peak at 80 to 100 days. After a short plateau period of about one month it declined to low levels at 150 to 200 days. Re-exposure of these calves when the titre had declined produced a rapid rise in antibody levels characteristic of a secondary response and reaching a peak in 3 to 4 weeks.

This paper presents the results of studies using the same 'boiled' antigen as Jarrett *et al.*, comparing the results with those obtained by Weber with the low temperature antigen and extending the observations to calves infected out-of-doors.

METHODS

Experimental animals. Calves were reared worm-free indoors by methods previously described (Michel, 1954). The parasitological techniques employed included faecal larval counts, estimation of infective larvae on pasture, culture of infective larvae for experimental infection and recovery of worms from affected lungs. The details of these methods were recorded in earlier publications (Michel 1954; Parfitt 1955; Michel and Parfitt 1955).

Complement fixation. The tests were carried out on the 50 per cent. haemolysis end-point basis using a serum dilution method. A 3 per cent. suspension of sheep red blood cells standardised by means of an E.E.L. colorimeter was used both in the test and to prepare a set of standards reading from 10 to 100 per cent. against which degrees of haemolysis could be read visually. Complement was titrated daily and three 50 per cent. units employed in the test and one 50 per cent. unit in the controls. The antigen was prepared from adult worms by the method of Stewart (1950). The results were read after the tubes had been left standing overnight at 4°C and the titre of each serum expressed as the reciprocal of the dilution at which the end point was reached. Adequate controls including standard known negative and positive sera were set up with each test.

Indoor experiments. Six calves were given a single dose of 3,000 to 3,500 larvae (Table 1). A further calf (3840) was given 6 doses of larvae varying from 5,000 to 20,000 at intervals of 6 to 10 weeks.

Outdoor experiments. The general pattern of outdoor infections was that in most cases, a calf was given a dose of larvae and put onto a lungworm-free pasture; then at different intervals other calves were added to or removed from the pasture (21 calves in all). The source of the infection on the pasture grazed by two other calves (244 and 245) was derived from the boots of workers traversing it from infected fields. Finally, calves 2700 and 3381 had already been exposed to infection and were then kept indoors for 20 and 11 months respectively and finally re-exposed for a period of 18 days.

The epidemiological significance of the experiments involving calves 220, 237, 253, 267, 272, 244, 245, 2700 and 3381 has already been reported (Michel and Parfitt, 1956; Michel 1957).

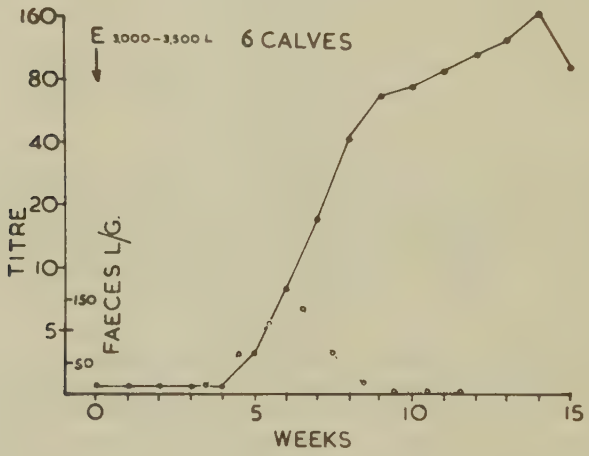
In all experiments blood samples were collected weekly or fortnightly. Faeces and herbage samples were taken weekly.

RESULTS

Indoor Experiments

The mean antibody levels and faecal larval output of the 6 calves receiving a single dose of larvae are shown in Fig. 1. Antibodies were first detected about 5 weeks after the initial infection and reached a peak 9 weeks later. Faecal larval output preceded the onset of detectable antibody production by about 2 weeks, reached a peak while the titre was still low and ceased with elimination of the infection before the titre had reached its peak level. The individual and mean times for the commencement of titre and the peak of antibody production are shown in Table 1. The details of calf 3840 which received multiple doses are presented in Fig. 2. Circulating antibody was first detected 3 weeks after the initial dose of 5,000 larvae and reached a peak 6 weeks later, 2 weeks after the second dose. There followed a short plateau period and then the titre, although fluctuating, declined steadily in spite of further doses of larvae to which the calf was completely immune. No true secondary response could be detected in this animal.

Fig. 1



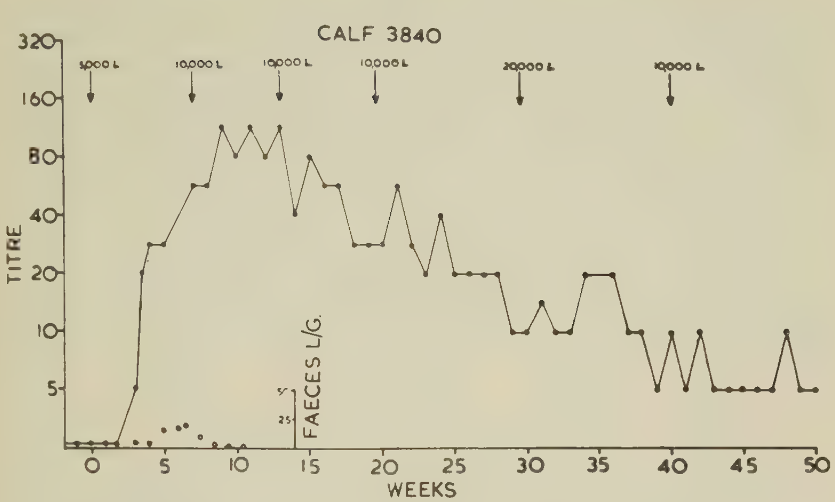
Mean antibody titre and mean faecal larval output of 6 calves dosed with 3,000 to 3,500 larvae. E = time of exposure.
●——● = antibody titre, o . . . . . o = larvae in faeces.

TABLE 1

INDIVIDUAL AND MEAN TIMES FOR THE COMMENCEMENT OF ANTIBODY TITRES AND THE ATTAINMENT OF THE PEAK RESPONSE IN 6 CALVES AFTER AN INITIAL DOSE OF 3,000–3,500 LARVAE.

Calf	*Days to onset of titre*	*Days to peak titre*
129	49	98
171	39	84
164	33	60
168	33	67
175	25	82
1009	33	69
Mean	35·3	76.6

Fig. 2



Antibody titre and faecal larval output of calf receiving repeated doses of larvae. ●——● = antibody titre, o . . . . . o = larvae in faeces.

TABLE 2

SEROLOGICAL AND PARASITOLOGICAL DATA

6 calves given a dose of larvae and put onto pasture to act as carriers

Calf	*Initial dose of larvae*	*Days to onset of titre*	*Days to peak titre*	*Pre-patent period (days)*	*Patent period (days)*	*Fate and titre**	*Worms in lungs*
227	2,000	21	—	27	28	D(76) 160	992
228	2,000	48	146	21	91	—	—
229	2,000	42	76	21	69	K(384) 160	0
253	10 + 30	47	126	26	63	K(224) 80	63
220	40	34	—	26	—	D(72) 40	2021
237	40	47	—	26	—	D(66) 10	2159
Mean	—	40	116	24·5	63	—	—

K = Killed D = Died

* Figures in brackets represent days to death or slaughter.

Outdoor Experiments

The 6 calves which were dosed with larvae to act as carriers to infect the various pastures, received from 40 to 2,000 larvae (Table 2). The first antibody response occurred 21 to 48 days after infection and bore no relation to the size of the infecting dose. In the 3 calves which survived the ensuing herbage infestation the peak titre was reached in 76 to 146 days. Of 14 calves which were put directly onto the infected pastures (Table 3) the onset of the titre in 12 of them occurred at 20 to 50 days, with a mean of 31 days from the day of exposure. The peak titre in the 5 calves which survived long enough was reached in 27 to 136 days with a mean of 79 days. Two calves died before antibody levels could be detected, and one of these (426) had no titre at the time of death 10 weeks after exposure. Nine animals died after the titre had begun to develop but while it was evidently still increasing and one calf died after the titre had reached its peak. It will be seen that there is no connection between the titre at the time of death or slaughter and the numbers of worms in the lungs.

TABLE 3

SEROLOGICAL AND PARASITOLOGICAL DATA

14 calves put onto infected pasture

Calf	*Days to onset of titre*	*Days to peak titre*	*Pre-patent period (days)*	*Patent period (days)*	*Fate and titre**	*Worms in lungs*
279	27	—	27	—	D(36) 5	1792
317	48	98	27	55	K(316) 80**	0
318	34	—	20	—	D(43) 10	4644
368	—	—	27	—	D(27) 0	7893
426	—	—	27	—	D(66) 0	997
427	20	—	27	—	D(29) 5	2958
447	34	—	34	—	KE(83) 20	597
464	27	—	27	—	D(66) 40	1124
471	53	95	25	70	K(126) 160	0
522	27	—	27	64	—	—
562	20	27	27	—	AD(59) 160	648
563	20	41	27	—	D(72) 640***	618
267	38	136	25	84	K(187) 80	37
272	25	—	25	—	D(68) 60	2159
Mean	31	79	26·5	68	—	—

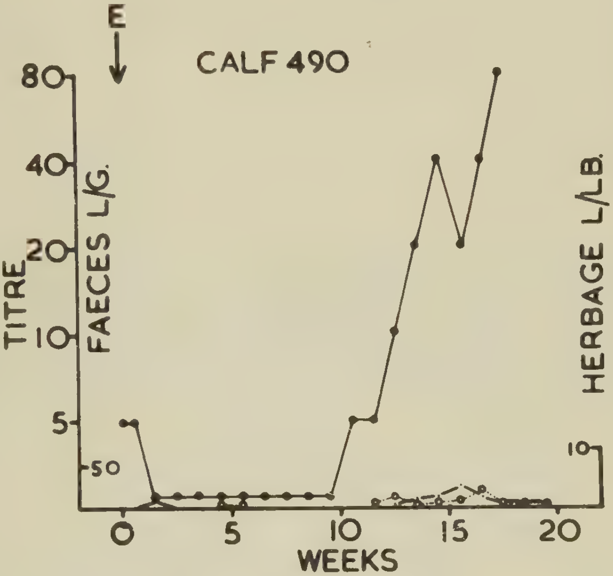
* Figures in brackets represent days to death or slaughter.

** Titre 60 days before slaughter. *** Titre 30 days before death.

K = Killed. *D* = Died. AD = Accidental death. KE = Killed in extremis.

Calf 490, which is not included in Table 3, experienced only a small, transient herbage infestation which was reflected in the development of a small degree of patency, also of a transient nature. Antibody production did not take place until a week before larvae re-appeared in the faeces (Fig. 3). This second period of patency also produced a second rise in herbage infestation.

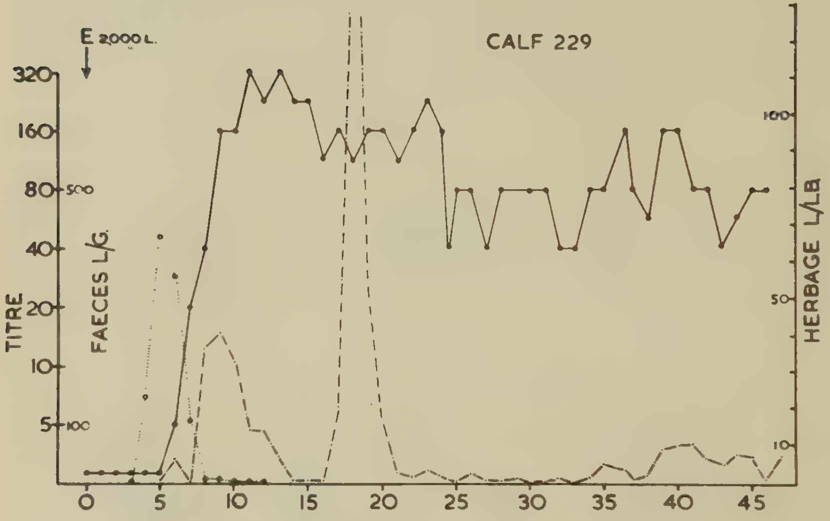
Fig. 3



Antibody titre and faecal larval output of grazing calf.
E = time of exposure, ●——● = antibody titre, o . . . . o = larvae in faeces, .—.—.—. = larvae on herbage.

The pattern of antibody response after the peak level has been passed is illustrated in Figs. 4 and 5 where the titre remained in the middle range, having fallen from the maximum. This was the case as long as the herbage remained infective, and the sudden high herbage infestation experienced by calf 229 (Fig. 4) after the peak titre had been reached did not alter this pattern. When the pasture lost its infectivity, as in the case of calf 317 (Fig. 6), the titre fell to a low level, but rose again immediately the pasture became reinfected. The antibody levels in calves 2700 and 3381, which had been kept indoors 20 and 11 months respectively after their first infection, are shown in Fig. 7. A low residual titre was present in these two animals as a result of the previous exposures, and re-infection produced a true secondary response which began at 7 days and reached a peak 21 to 28 days later. The data relating to calves 244 and 245, which were grazing a field subjected to contamination by worker' boots, are shown in Figs. 8 and 9. It is not possible to determine the interval

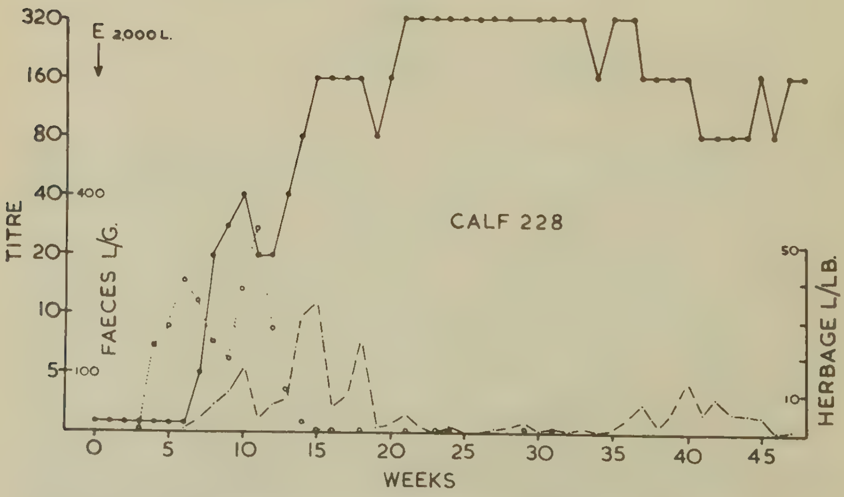
Fig. 4



Antibody titre and faecal larval output of calf given 2,000 larvae and put onto pasture at time E.

●——● = antibody titre, o . . . . . o = larvae in faeces, .—.—.—. = larvae on herbage.

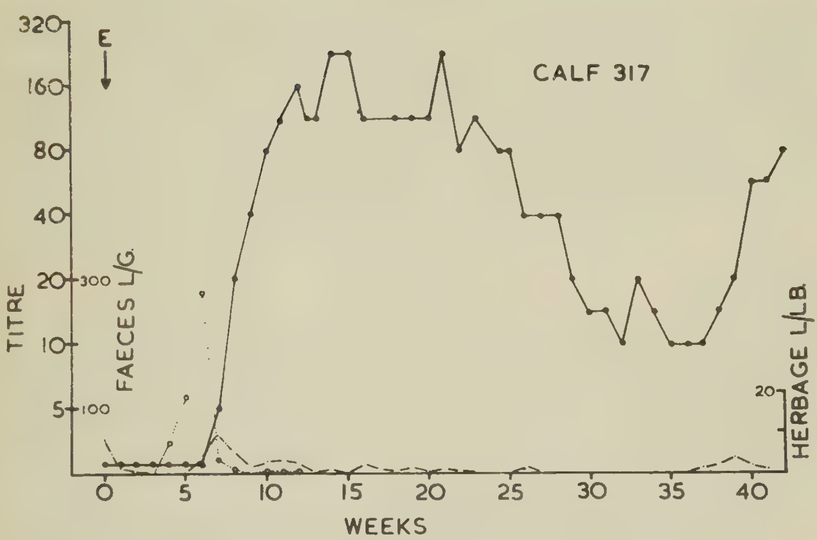
Fig. 5



Antibody titre and faecal larval output of calf given 2,000 larvae and put onto pasture at time E.

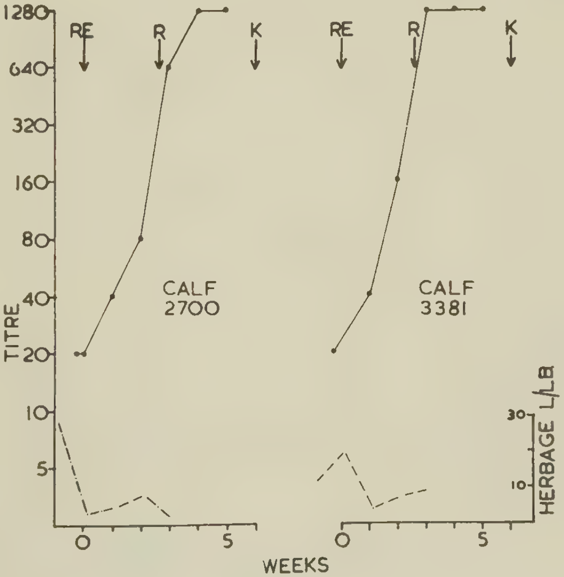
●——● = antibody titre, o . . . . . o = larvae in faeces, .—.—.—. = larvae on herbage.

Fig. 6



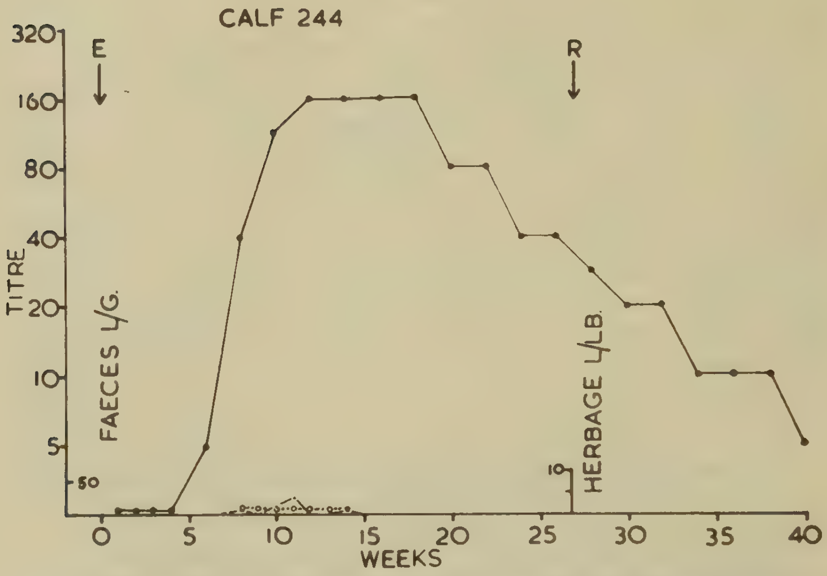
Antibody titre and faecal larval output of calf grazing infected pasture. ●——● = antibody titre, o . . . . . o = larvae in faeces, .—.—.—. = larvae on herbage.

Fig. 7



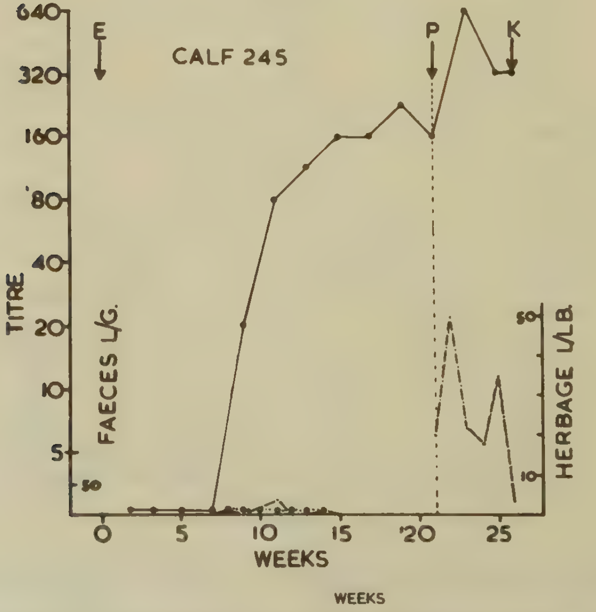
Antibody titres of two calves re-exposed on infected pasture after a period indoors.
●——● = antibody titre, .—.—.—. = larvae on harbage, RE = re-exposure. R = removed, K = killed.

Fig. 8



Antibody titre and faecal larval output of grazing calf. Pasture exposed to infection from time E.
●——● = antibody titre, o . . . . . o = larvae in faeces, .—.—.—. = larvae on herbage. R = removed from pasture.

Fig. 9



Antibody titre and faecal larval count of grazing calf. Pasture exposed to infection from time E.
●——● = antibody titre, o . . . . . o = larvae in faeces, .—.—.—. = larvae on herbage. P = transfer to different pasture. K = killed.

between exposure and the onset of antibody production because the exact time of infection is not known, but the titre commenced 2 weeks before faecal and herbage samples were positive in 244 and coincided with them in 245. The peak titres were reached in 42 to 69 days after the onset. Calf 244 was later removed from the pasture and its titre which had already begun to decline, fell to a low level while indoors. Calf 245 still had a well developed titre when it was transferred to a heavily infected pasture, but a further rise in antibody levels was stimulated by this challenge exposure.

DISCUSSION

The results of the indoor experiments with single doses of larvae are in close agreement with those of Jarrett *et al.* (1959) regarding the appearance of antibodies and their subsequent rise to a peak. The discrepancy between our results and those reported by Weber (1958) is not easy to explain. The latter suggested that the small size of the initial dose (500 to 1,500 larvae) given to his calves which were re-exposed once may account for the lack of any difference between these calves and those exposed to larger initial doses of larvae (5,000 to 50,000). Whether this is so or not the early onset of antibody production at 12 days and the attainment of a peak response 4 days later is more typical of a secondary than of a primary response. Another possible explanation of Weber's results may be that he used an antigen which was prepared by a low temperature method and so might well differ in antigenic structure and composition from the heated antigen used in the present studies. Cornwell (1959), however, compared these two types of antigen and found no difference in the time of onset of antibody titre in the same serum samples. The results in calf 3840 (Fig. 2) suggest that repeated doses of larvae at intervals of 6 to 10 weeks fail to 'boost' the titre in the manner of a secondary response, and although the calf is resistant to the repeated challenge the titre steadily declines. In Weber's multiple exposure group he similarly failed to 'boost' the titre but found no decline in antibody level. Jarrett *et al.* (1959) found a typical secondary response when calves were reinfected at intervals of 150 and 160 days. It may be that the interval between initial exposure and re-exposure must be sufficiently long to allow the titre to decline if a typical secondary response is to be obtained. There may also be a limit to the number of exposures which will produce a secondary rise in titre although this might depend on the interval between them.

We suggest that the stimulus to antibody production which the heated whole worm antigen detects may be the arrival and development of immature worms in the lungs. If this is so one would expect that after an interval of several months—a period long enough to allow the resistance of the host to decrease and so allow some worms of a challenge dose to become established in the lungs before being destroyed or eliminated—the titre will rise in response to exposure. Repeated exposures at shorter intervals, however, would result in few or no worms of the later doses becoming established and no stimulation of antibodies.

The results of the outdoor experiments show no essential difference in the pattern of antibody response from that in the indoor calves given a single dose of larvae. The continuous acquisition of infective larvae on the herbage results in the maintainance of a fairly uniform titre in the middle range. If the herbage infestation falls to a level undetectable by the method of estimation used, the titre of the grazing calf commences to fall and remains at a low level unless the herbage again shows a detectable number of larvae, when the titre rises in a manner typical of a secondary response (Fig. 6). A similar picture is seen when a calf is removed entirely from the pasture for several months and then re-exposed to infected herbage (Fig. 7). An intermediate situation is seen to occur with calf 245 (Fig. 9) which showed a four-fold rise in titre when transferred to a heavily infected pasture though its titre was moderately high at the time. This probably occurred because the calf had experienced no detectable herbage infestation for 8 weeks prior to transfer. Under conditions of continuous exposure the pattern of the response is unaffected by a sudden increase in herbage infestation, as evidenced by calf 229 (Fig. 4). Calf 490 (Fig. 3) is deserving of special mention since the acquisition of a small but temporary patent infection from a transient herbage infestation was insufficient to stimulate detectable levels of antibody until about the time larvae re-appeared in the faeces and produced a further rise in the herbage infestation. No firm conclusions can be drawn from this one animal but it seems reasonable to assume that a certain minimal level of larval intake is necessary to produce an antibody response. It was reported by Michel and Cornwell (1959) that no relationship exists between the resistance of a calf to the establishment of a second infection and the complement-fixing titre at the time of challenge. The results presented here suggest that the development of antibodies is related to the time elapsing since the initial exposure and not to the clinical severity of the disease, the fate of the calf, or the worm burden at post mortem.

CONCLUSIONS

Complement fixation tests using a heated adult worm antigen were carried out on the sera of 6 calves infected indoors with a single dose of *Dictyocaulus viviparus* larvae, one calf given multiple doses of larvae indoors and 24 calves exposed to infection at pasture. Faecal larval counts, herbage counts and post mortem worm counts were carried out. There was little difference between the indoor and outdoor experiments in the time of onset of the antibody response or the time taken to reach a peak. The time elapsing was 35 days to onset and 76 days to the peak for the indoor calves and 31 and 79 days, respectively, in the outdoor calves. In the presence of a continuous herbage infestation the titre remains in the middle range after falling from the peak. Should the herbage infestation cease or the calf be removed from the pasture the titre falls to a low level, but when re-exposed to infection a secondary response occurs in 7 days, reaching a peak in 21 to 28 days. One calf given repeated doses of larvae at intervals of 6 to 10 weeks did not show a true secondary response and its titre declined to a low level in spite of the repeated infections. It is suggested that a secondary response can occur when worms become established in the lungs of an animal which has had previous experience of infection but has either not acquired full protection or, in the absence of re-infection, has lost it. The entirely refractory calf gives no such response because no parasites become established.

ACKNOWLEDGMENTS

We are indebted to Professor E. G. White for his interest in this work. Thanks are also due to Miss J. Berry for technical assistance. One of us (R.L.C.) is grateful to the Agricultural Research Council for financial assistance.

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THE COMPLEMENT-FIXING ANTIBODY RESPONSE OF CALVES TO *DICTYOCAULUS VIVIPARUS*

II. Vaccination with Irradiated Larvae

By

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INTRODUCTION

Interest has in recent years been focused on lungworm disease of cattle because of its annual toll of bovine lives and the lack of an effective treatment.

In their immunological studies on *D. viviparus* which led to the production of a vaccine consisting of irradiated infective larvae, Jarrett, Jennings, McIntyre, Mulligan, Thomas and Urquhart (1959) used a quantitative complement fixation test and a heated whole worm antigen. Cornwell and Michel (1960), using the same antigen as Jarret *et al*, obtained results with calves infected indoors and at pasture which were in agreement with them, but not with Weber (1958), who used a frozen antigen. Michel and Cornwell (1959) reported that no correlation could be demonstrated between the resistance of calves to the establishment of the worms of a second exposure and the complement-fixing antibody titre at the time of this challenge. Present knowledge is insufficient to indicate what part of the life cycle stimulates these non-protective antibodies which fix complement with the whole worm antigen.

Irradiated larvae of *D. viviparus* are only capable of a partial migration in the body and most of them fail to reach the lungs (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1957).

The antibody response of calves to vaccine might thus be different from that to normal larvae and might help to throw some light on the stage of the parasite responsible for the antibodies which are being measured. Serological studies on calves vaccinated with irradiated larvae were therefore made and the findings are reported here.

METHODS

A total of 34 Ayrshire calves on 2 different farms were bucketfed and reared indoors from birth in loose-boxes. They were not regularly cleaned out. On Farm A a total of 19 calves varying in age from 2 to 6 months were divided into four groups: 10 were given 2 doses of irradiated *D. viviparus** larvae with an interval of 6 weeks between doses, 4 calves were dosed at an interval of 4 weeks, and 4 at an interval of 11 weeks. One calf was left unvaccinated.

On Farm B there were 15 calves varying in age from 2 to 11 months. One calf was left unvaccinated and the rest were given 2 doses of vaccine, with an interval of 6 weeks between doses.

Calves on both farms were bled weekly or fortnightly, beginning before the first dose of vaccine, and faeces samples were examined at intervals by Baermann extraction. The calves were turned out to grass not less than 4 weeks after the second dose of vaccine.

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* "Dictol", Allen & Hanburys Ltd.

Complement fixation tests were carried out as previously reported (Cornwell and Michel 1960).

TABLE 1

TITRES OF CALVES RE-VACCINATED 6 WEEKS AFTER THE FIRST DOSE

	Calf no.	*Age mnths.*	*Weeks after first dose of vaccine*					
			*0**	*2*	*4*	*6†*	*8*	*10*
FARM B	627	4	0	0	5	10	80	160
	146	7	0	0	10	10	30	120
	215	2	0	0	0	0	20	120
	430	7	0	0	0	5	40	40
	226	10	0	0	5	10	20	40
	184	3	0	0	0	0	5	40
	211	7	0	0	0	0	10	30
	212	4	0	0	0	0	5	30
	832	11	0	0	0	0	20	10
	515	7	0	0	0	0	0	5
	634	5	0	0	0	0	0	5
	521	5	0	0	0	0	0	0
	848	10	0	0	0	0	0	0
	518	2	0	0	0	0	0	0
FARM A	300	3½	0	0	5	15	160	160
	228	4	0	5	40	20	80	160
	517	4½	0	0	20	20	80	160
	418	5	0	0	5	5	10	120
	609	4	0	0	5	5	60	80
	512	4½	0	0	30	20	80	80
	330	3½	0	0	5	5	15	80
	739	4½	0	0	0	0	40	60
	240	3½	0	0	5	0	5	40
	239	3½	0	0	0	0	5	0

* 1st. dose of vaccine.
† 2nd. dose of vaccine.

RESULTS

The antibody response of the 24 calves with an interval of 6 weeks between the 2 doses of vaccine is shown in Table 1. At the time the second dose of vaccine was given thirteen calves had not developed any titre and the remaining 11 calves had titres ranging from 5 to 20. The titre then rose to a mean of 42 on Farm B and 94 on Farm A a month after the second dose of vaccine, when the animals were turned out to grass. The response was more uniform on Farm A and the titres, except for the one calf which responded only temporarily to a titre of 5, varied from 40 to 160. The calves on Farm B developed titres from 0 to 160. Three calves failed to develop detectable antibodies.

The results for the four calves with an interval of 4 weeks between doses are shown in Table 2. In this group a response to the first dose was evident as early as 2 weeks and the mean titre was 40 at the time of the second dose. At the time of going out to grass the mean titre had risen to 95.

The group of 4 calves with an interval of 11 weeks between doses (Table 3) showed a neglible response to the first dose. After the second dose one calf developed a moderate titre of 60, two calves had low titres of 10 to 20 and the remaining calf did not respond at all. The two control calves developed no antibody titre prior to being turned out to graze. No positive faeces were detected and no coughing occurred in the vaccinated animals during this period.

TABLE 2

TITRES OF CALVES RE-VACCINATED 4 WEEKS AFTER THE FIRST DOSE

Calf no.	*Age mnths.*	*Weeks after first dose of vaccine*								
		0*	*1*	*2*	*3*	*4*†	*5*	*6*	*7*	*8*
836	6	5	5	40	30	80	80	160	160	160
301	6	0	0	30	40	30	30	80	80	120
125	5	10	10	20	60	30	30	30	80	80
073	6	0	0	5	10	20	20	10	20	20

* 1st. dose of vaccine.
† 2nd. dose of vaccine.

TABLE 3

TITRES OF CALVES RE-VACCINATED 11 WEEKS AFTER THE FIRST DOSE

Calf no.	*Age mnths*	*Weeks after first dose of vaccine*														
		*0**	*2*	*3½*	*5*	*6*	*7*	*8*	*9*	*10*	*11*†	*12*	*13*	*14*	*15½*	*17*
217	3	0	0	0	0	0	0	5	5	10	5	5	5	20	60	60
216	2	0	0	0	0	0	0	0	5	0	10	10	5	20	20	10
538	2	0	0	0	0	5	5	5	10	5	5	0	0	0	15	10
938	3	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0

* 1st. dose of vaccine.
† 2nd. dose of vaccine.

DISCUSSION

These results show that the antibody response to the first dose of vaccine is usually of a low order. A similar titre of 5 or even 10 is found occasionally in calves which have not been exposed to infection. It is only possible to judge the full response to a single dose in the 11-week interval group since the other groups received the second dose before there had been sufficient time for the response to the first to develop fully. The second dose of vaccine usually "boosted" the titre, but the response of individual calves varied widely and 3 out of 32 calves failed to develop a detectable response to either dose. Two calves of the 4-week interval group showed low titres before vaccination and the titres of all 4 calves began to rise 2 weeks after the first dose of vaccine. This type of response is unusually early and calves given normal larvae do not show such an early response (Jarrett *et al.*, 1959; Michel and Cornwell, 1959). It may be that these 4 calves, which were the oldest group on Farm A and were housed together in a loose box away from the other calves, had experienced a degree of lungworm or other infection indoors sufficient to sensitize them to a dose of irradiated larvae and so provoke an early rise in titre.

It is pertinent to enquire into the reason for the wide variation in the antibody response, some calves not even reacting to the second dose. It is known that some of the irradiated larvae reach the lungs and produce lesions (Jarrett *et al.*, 1959) but no estimate of the frequency or extent to which this occurs has been reported. It seems probable that in some calves no larvae succeed in migrating to the lungs, whereas in others they do so and develop for a short time before dying or being expelled.

Normal larvae produce an antibody response commencing in 4 to 6 weeks when given to calves in a single dose (Jarrett *et al.*, 1959; Michel and Cornwell, 1959). Irradiated larvae also produced an antibody response in 4 to 6 weeks in 11 of 24 calves in the 6-week interval group (Table 1). It seems reasonable therefore to assume that in these calves the irradiated larvae provided the same sort of antigenic stimulus as normal larvae. It is suggested that the antibody response measured here may result from larvae becoming established in the lungs, and that the failure of a calf to develop antibodies indicates the failure of larvae to complete their migration.

Although it was necessary to rely on the observations of the stockmen, no coughing was noticed among the calves throughout the vaccination procedure. All faeces samples were negative for lungworm larvae and so there was no evidence that irradiated larvae matured sufficiently to produce larvae in the faeces. The 11-week interval group were the youngest calves in the experiment and showed the lowest titres, but no relationship was found in the other groups between the age of a calf and the titre it developed.

CONCLUSIONS

The antibody response of 32 calves vaccinated with a double dose of irradiated *D. viviparus* larval vaccine was measured by the complement fixation test, using a whole worm antigen. Titres resulting from the first dose were low, while the second produced a variable rise in titre and at the time of going out to grass 4 to 5 weeks later the antibody levels were in the low or middle range. Individual calves showed a wide variation in response and in 3 calves no antibodies were produced by either dose. It is suggested that the antibody response may be due to irradiated larvae which succeed in becoming established in the lungs before being eliminated. No evidence was found that irradiated larvae develop to maturity.

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THE COMPLEMENT FIXING ANTIBODY RESPONSE OF CALVES TO *DICTYOCAULUS VIVIPARUS*

III. Vaccinated Calves Exposed to Challenge

By

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INTRODUCTION

The use of X-rays to attenuate *Dictyocaulus viviparus* larvae in the production of a vaccine against parasitic bronchitis has been described by Jarrett, Jennings, McIntyre, Mulligan and Urquhart (1957). Later reports of field trials using a single dose of vaccine have been promising (Jarrett, Jennings, McIntyre, Martin, Mulligan, Sharp and Uquhart (1958) and still better protection was obtained with two doses of vaccine in calves challenged with 10,000 larvae (Jarrett, Jennings, McIntyre, Mulligan, Sharp and Urquhart 1959a).

The use of the complement fixation test to measure levels of circulating antibody in calves experimentally infected with *D. viviparus* has been reported by Weber (1958) who prepared his adult worm antigen by a low temperature method. Jarrett *et al.* (1957), Jarrett, Jennings, McIntyre, Mulligan, Thomas and Urquhart 1959b and Cornwell and Michel (1960) using a heated adult worm antigen obtained results in close agreement with each other but not with Weber. These results are discussed by Cornwell and Michel (1960).

The complement fixing antibodies measured by the whole worm antigen give no true indication of the ability of a calf to prevent the establishment of the worms of a challenge dose (Cornwell and Michel, 1959) and it is not known what stage of the parasite is responsible for the stimulation of these non-protective antibodies.

Cornwell (1960) studied the response of calves given two doses of irradiated *D. viviparus* larvae* and found that a negligible titre resulted from the first dose: whilst the second dose usually "boosted" the titre, a wide variation existed between individual calves 3 out of 32 calves failing to show demonstrable antibodies to either dose.

The present paper is concerned with the continuation of studies on vaccinated calves when exposed to challenge on infected pasture and experimentally indoors.

MATERIALS AND METHODS

Details of the calves used in these observations have been described (Cornwell, 1960). In all calves the first dose of vaccine was given on 19th March, 1959.

In all experiments, except the first, faeces samples were taken regularly and examined by the following method:—Ten grammes of rectal faeces were placed in a Baermann funnel with water at 37°C. and left on the bench overnight. Sufficient fluid to fill a centrifuge tube was then run off and spun at 1500 r.p.m. for 2 minutes. The supernatant was siphoned off and the sediment transferred to a microscope slide and the larvae counted. This method regularly detected small numbers of larvae being passed over periods of several weeks.

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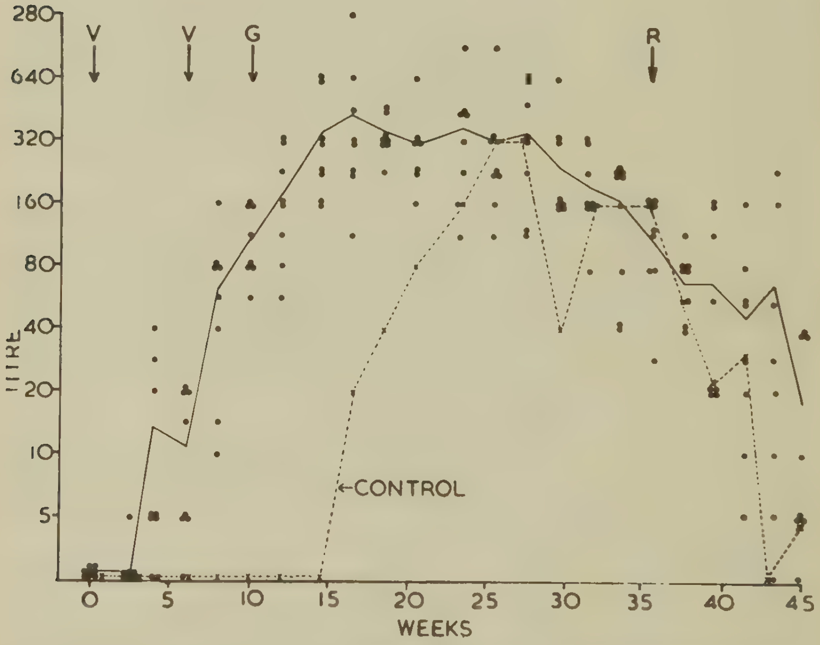
* "Dictol", Allen & Hanbury's Ltd.

Blood samples were taken weekly or fortnightly and complement fixation tests carried out on the serum using the method previously described (Cornwell and Michel, 1960).

Pasture samples for the estimation of numbers of infective larvae on the pasture at the Field Station were examined by the method of Michel and Parfitt (1955).

Fig. 1



Individial and mean antibody titres of 8 vaccinated calves and one unvaccinated control calf. Vaccination interval 6 weeks.
V = dose of vaccine given. G = exposure to infected pasture.
R = removal from pasture.

RESULTS

Farm A

Experiment 1. Eight calves vaccinated with an interval of 6 weeks between the two doses were turned out to grass along with an unvaccinated control calf 4 weeks after the second dose. The antibody levels of these calves are shown in Fig. 1. The control calf 134 (Fig. 1 and Table 1) showed symptoms and on faeces examination 7 weeks after exposure 42 larvae per g. were detected. Thereafter the count fell and was negative 9 weeks later. The antibody titre of this calf commenced 6 weeks after exposure, reaching a peak in 15 weeks. The titre then fluctuated until the calf was housed when it fell to a low level. This control calf showed that the pasture was infected and constituted a challenge to the vaccinates. The mean titre of the 8 vaccinates was 112 at the time of going out to grass and it continued to rise to a peak of 455 six weeks later. The antibody level was maintained for some weeks until the pastures became bare at the end of the grazing season when a fall in titre was evident, and continued when the animals were housed. This group showed mild symptoms of husk and at the time of a single faeces examination 8 weeks after going out to grass 5 out of the 8 calves were passing small numbers of larvae, the highest count being 3·7 l/g.

TABLE 1

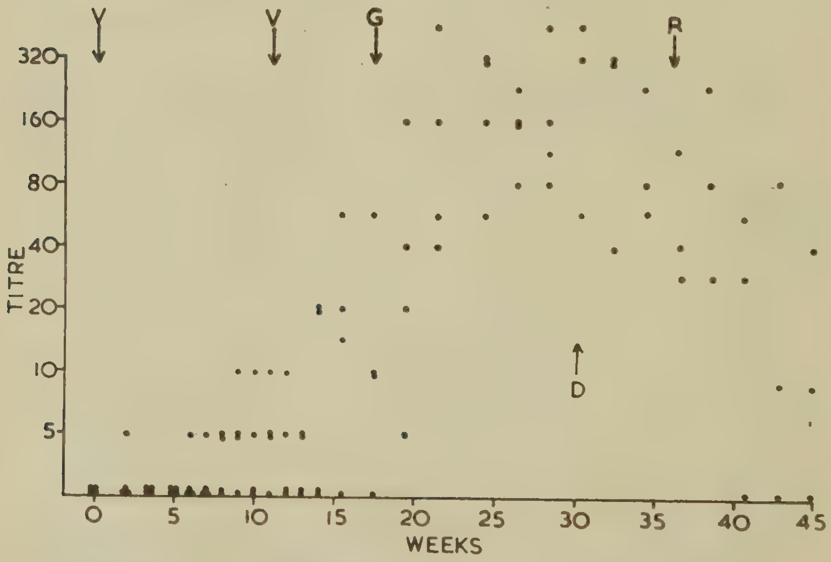
ANTIBODY TITRES AND FAECAL LARVAL OUTPUT OF 4 CALVES GIVEN TWO DOSES OF VACCINE WITH AN INTERVAL OF 11 WEEKS BETWEEN DOSES (EXPERIMENT 2) AND ONE UNVACCINATED CONTROL CALF (EXPERIMENT 1)

Calf	*216*		*217*		*538*		*938*		*134 (Control)*	
Date	*Titre*	*Faeces (l/g*	*Titre*	*Faeces (l/g)*	*Titre*	*Faeces (l/g)*	*Titre*	*Faeces (l/g)*	*Titre*	*Faeces (l/g)*
17. 3.59	0		0		0		0		0	
V 19. 3.59	0		0		0		0		0	
26. 3.59	0		0		0		5		0	
6. 4.59	0		0		0		0		0	
10. 4.59	0		0		0		0		0	
17. 4.59	0	0	0	0	0	0	0	0	0	0
24. 4.59	0		0		5		0		0	
1. 5.59	0		0		5		0		0	
8. 5.59	0	0	5	0	5	0	0	0	0	0
15. 5.59	5		5		10		0		0	
23. 5.59	0		10		5		0		0	
28. 5.59	10	0	5	0	5	0	0	0	0	0
V 2. 6.59	N.S.		N.S.		N.S.		N.S.		N.S.	
5. 6.59	10		5		0		0		G 0	
12. 6.59	5		5		0		0		0	
19. 6.59	20	0	20	0	0	0	0	0	0	N.S.
29. 6.59	20		60		15		0		0	N.S.
G 13. 7.59	10		60		10		0		20	N.S.
27. 7.59	40	0	160	0	5	0	20	0	40	42.7
10. 8.59	160	0.4	480	0	40	1.2	60	0.1	80	21.0
31. 8.59	320	N.S.	320	2.7	160	11.7	60	15.2	160	1.2
14. 9.59	160	1.7	240	0	160	138	80	15.2	320	0.9
28. 9.59	80	0.1	480	0	160	212	120	27.0	320	0
14.10.59	60	0.6	480	0.4	Died		320	4.4	160	0
27.10.59	40	0.6	320	0			320	13.6	160	0
9.11.59	80	0.2	240	0			60	2.2	160	
R 23.11.59	30	0.2	120	0			40	4.1	160	
8.12.59	30	0.3	240				80	2.4	60	
22.12.59	5	0	80				40	0	20	
4. 1.60	0	0	30				60	0.1	30	
18. 1.60	0	0	80				10	0	0	
25. 1.60	0		40				10	0	5	

V — Vaccine given
R — Removal from pasture
G — Date of exposure to infected pasture
N.S. — No sample

Experiment 2. Four calves vaccinated with an interval of 11 weeks between the two doses were turned out to grass 6 weeks after the second dose, and their faeces were sampled from the time of exposure until the larval count had returned to a consistently negative result. The titres of these calves are presented in Fig. 2. In response to the challenge the titres which had been insignificant in 3 calves all rose to a mean of 215 at the peak 6 weeks after exposure. The antibody level then remained steady, commencing to fall just before the calves were housed. Symptoms appeared in these animals and larvae were detected in the faeces, one calf (538) was killed in extremis and found to have 850 worms in its lungs. The titres and faecal larval output of these calves is shown in Table 1.

Fig. 2

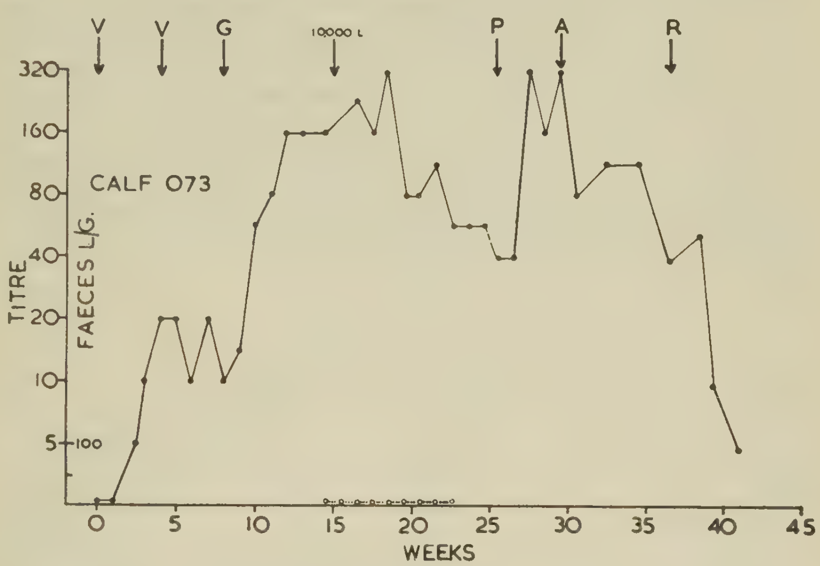


Antibody titres of 4 vaccinated calves. Vaccination interval 11 weeks.
V = dose of vaccine given. G = exposure to infected pasture.
R = removal from pasture. D = death of calf 538.

Experiment 3. Two calves vaccinated with an interval of 4 weeks between the two doses (073 and 125) and two calves with an interval of 6 weeks between doses (239 and 240) were turned out to grass 4 to 5 weeks after the second dose. On the 20th June they were transferred to the Field Station and put on to a lungworm-free pasture being grazed by unvaccinated calves.

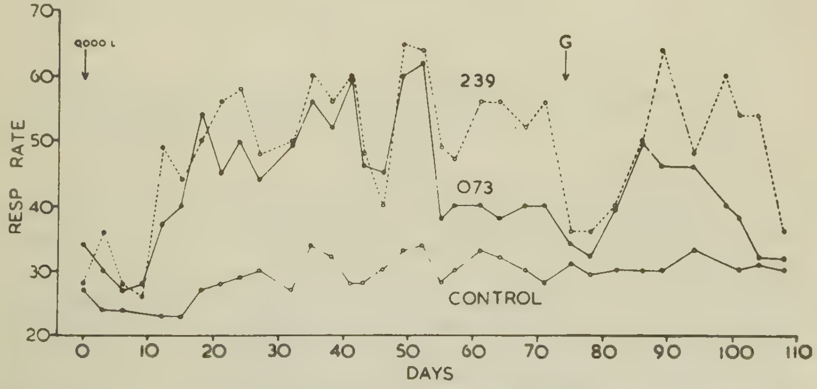
In experiment 3a, calves 073 and 239 were out at grass on Farm A 5 weeks and 2 weeks respectively before being brought to the Field Station and put on to the pasture that was being grazed by susceptible calves for 2 weeks. They were then brought indoors and given 10,000 larvae each. In addition to blood and faeces samples, respiratory rates were recorded. Ten weeks after this challenge dose they were turned on to an infected pasture for 5 weeks before returning to the farm of origin to be run with the main group of calves until housed for the winter.

Fig. 3



Antibody titre and faecal larval counts of calf 073.
V = dose of vaccine given. G = exposure to infected pasture at farm. 10,000 L = challenge indoors at Field Station. P = exposure to infected pasture. A = return to farm. R = removal from grass.

Fig. 4

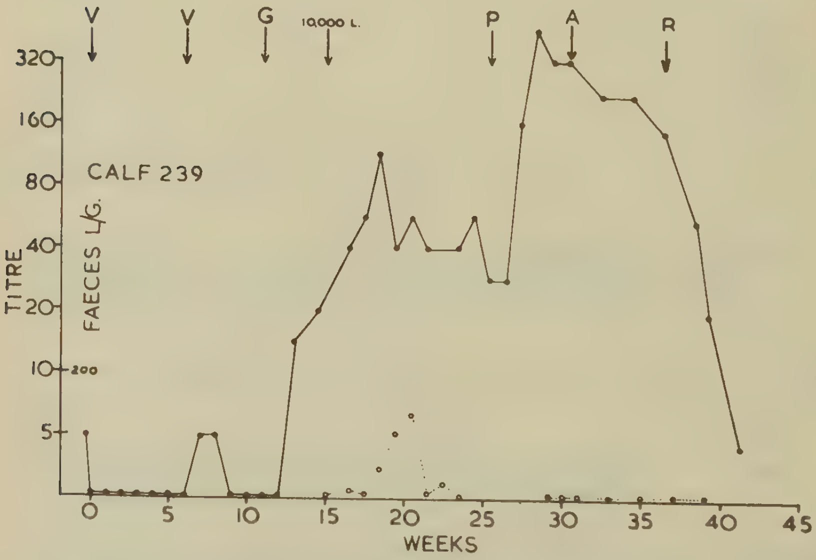


Respiratory rates of calves 073 and 239 compared with the mean of two unvaccinated uninfected control calves. 10,000 L = challenge dose indoors at Field Station. G = exposure to infected pasture.

Calf 073 (Fig. 3) developed a titre of 20 in response to vaccination and this was rapidly increased to 160 after exposure to the infected pasture on the farm. Larvae appeared in the faeces before the challenge dose was given and a low degree of patency was maintained for 8 weeks. The titre reached a peak of 320 after the challenge dose and then fell to 40 before re-exposure to infected pasture took place. A secondary response then occurred, the titre returning to 320 within 2 weeks. The titre again fell to 40 after the calf had returned home to the original pasture and dropped to zero after housing.

In response to the challenge dose this calf showed moderately severe symptoms of husk and a loss in weight occurred. The respiratory rate doubled and remained elevated until 50 to 60 days after challenge. A further rise in respiratory rate was produced by the infected pasture but no patency resulted. The respiratory rates of calves 073 and 239, compared with the mean of two uninfected controls, are shown in Fig. 4.

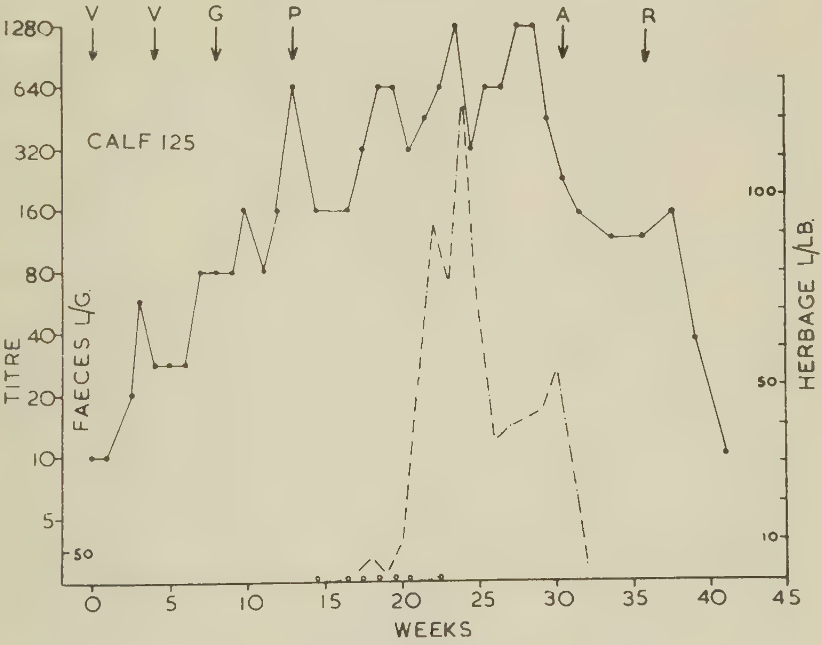
Fig. 5



Antibody titre and faecal larval counts of calf 239.
V = dose of vaccine given. G = exposure to infected pasture at farm. 10,000 L = challenge indoors at Field Station. P = exposure to infected pasture. A = return to farm. R = removal from grass.

Calf 239 (Fig. 5) developed no significant titre after vaccination, but an immediate response was seen on exposure to the pasture on the farm. The challenge dose was given when the titre was between 20 and 40 and a peak of 120 was produced before a falling off occurred. Exposure to infected pasture then resulted in a secondary response to a peak of 480, and then the titre gradually fell after the calf returned to the farm and was brought indoors. The faeces of this calf were positive on the day the challenge dose was given and the faecal output reached 131 l/g. The patent period lasted about 8 weeks. The symptoms shown by this calf were moderately severe and reference to Fig. 4 will show the course of the respiratory rate which was similar to that of 073 but slightly more prolonged. The later exposure to infected pasture resulted in a further increase in respiratory rate and a further period of patency.

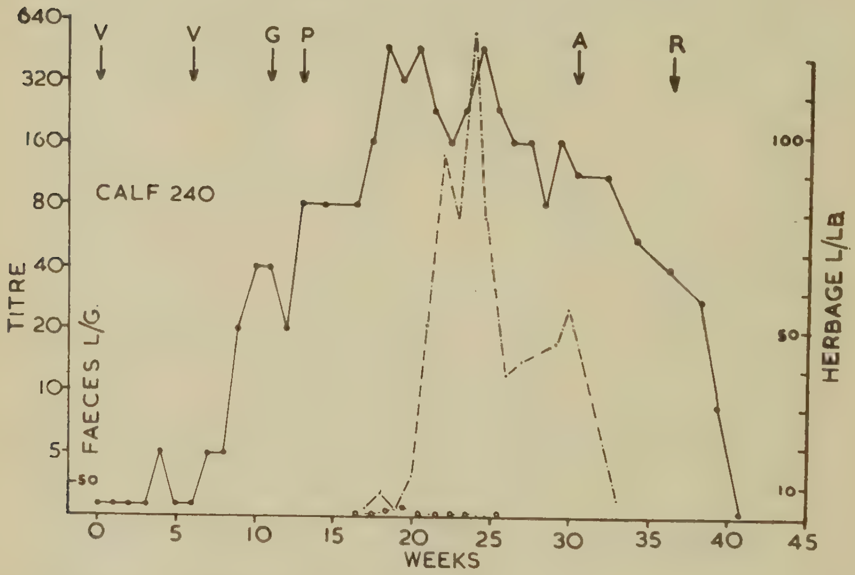
Fig. 6



Antibody titres and faecal larval counts of calf 125.
V = dose of vaccine given. G = exposure to infected pasture at farm.
P = transfer to uninfected pasture at Field Station. A = return to farm.
R = removal from grass.
●——● = antibody titre. .—.—.—. = numbers of infective larvae on the Field Station pasture. o . . . . . o = larvae in faces.

In experiment 3b, calves 125 and 240 remained on the pasture with 3 susceptible calves throughout the summer. One calf (125, Fig. 6) which had a titre of 10 before vaccination and one of 80 on exposure to the infected pasture at the farm showed a rise to 640 at the time of transfer to the Field Station. A week later the titre had dropped to 160 and the faeces commenced to show larvae. The patent period lasted 8 weeks and the titre rose to a peak of 1,280 as the pasture counts showed a rapid rise due to the larval excretion of unvaccinated calves. The titre remained high until the end of the summer when the calf was returned to the farm. The titre then began to fall and continued to fall when the animal was housed. A second calf (240, Fig. 7) had developed a titre of 40 when exposed to pasture on the farm and this titre rose to 80 at the time of transfer to the Field Station. The titre remained steady for 4 weeks and then rose to a peak of 640 as the pasture count similarly rose to a high level. A patent infection developed 38 days after the first exposure and lasted for 7 weeks. The titre had fallen to the middle range when the calf was returned home and the fall continued both at the farm and after housing for the winter.

Fig. 7



Antibody titres and faecal larval output of calf 240.
V = dose of vaccine given. G = exposure to infected pasture at farm.
P = transfer to uninfected pasture at Field Station. A = return to farm.
R = removal from grass.
●——● = antibody titre. .—.—.—. = numbers of infective larvae on Field Station pasture. o . . . . . o = larvae in faeces.

The pasture at Farm A which was grazed by all calves when first turned out had young adult cattle on it at the time. The calves which were transferred to the Field Station returned to this pasture on 20th October and all the calves were housed a month later.

Farm B

It was possible to continue observations on only 3 vaccinated calves and one unvaccinated calf on this farm.

Experiment 4. Two calves vaccinated with an interval of 6 weeks between the two doses of vaccine were turned into an orchard along with one unvaccinated calf 6 weeks after the second dose. They remained on the orchard for 11 weeks and were fed on hay, little grazing taking place. They were then transferred to a new ley, which had been grazed by adult cattle, where they remained for 8 weeks before being housed. At the time of going onto the orchard the titres of the vaccinates were 20 and 40. Thereafter they showed titres in the 80 range until transferred to the new ley following adult animals (Table 2). After exposure to the ley, one calf's titre rose to 160 and remained there until several weeks after housing. The titre of the other calf fell at first and then rose again to its original level until after housing. The control calf showed no titre nor were

TABLE 2

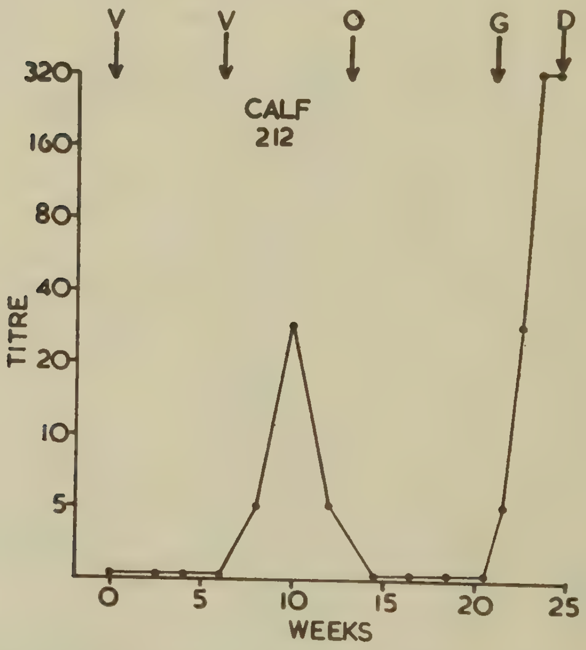
ANTIBODY TITRES AND FAECAL LARVAL OUTPUT OF 2 CALVES VACCINATED WITH TWO DOSES OF VACCINE AT AN INTERVAL OF 6 WEEKS AND ONE UNVACCINATED CALF (EXPERIMENT 4)

Calf	*184*		*215*		*397 (Control)*	
Date	*Titre*	*Faeces (l/g)*	*Titre*	*Faeces (l/g)*	*Titre*	*Faeces (l/g)*
17. 3.59	0		0		0	
V 19. 3.59	0		0		0	
6. 4.59	0		0		0	
17. 4.59	0	0	0	0	0	0
V 1. 5.59	0		0		0	
15. 5.59	5	0	20	0	0	0
28. 5.59	40		120		0	
12. 6.59	20	0	60	0	0	0
O 19. 6.59	N.S.		N.S.		N.S.	
29. 6.59	20		20		0	
13. 7.59	40		80		0	
27. 7.59	60	0	120	0	0	0
10. 8.59	80		80		0	
31. 8.59	80	0	80	0	0	0
G 7. 9.59	N.S.		N.S.		N.S.	
14. 9.59	80	0	60	0	0	0
28. 9.59	40	0	30	0	0	0
13.10.59	160	0.6	20	0.3	0	0.2
27.10.59	160	0.5	120	1.3	40	4.7
R 2.11.59	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
9.11.59	160	1.7	60	0.3	120	17.5
23.11.59	160	0	80	0	80	3.5
8.12.59	160	0	60	0	120	2.4
22.12.59	40	0	60	0	120	1.9
4. 1.60	20		160		160	0
18. 1.60	30		80		40	0
25. 1.60	10		20		10	0

V — Vaccine given
O — Turned into orchard
G — Turned onto ley
R — Removed from pasture
N.S. — No sample

larvae detected in the faeces until after grazing the ley pasture. This indicates that the calves did not meet infection until grazing the pasture vacated by the adult cattle and the appearance of larvae in the faeces of the two vaccinates and the control 37 days after exposure to this pasture supports this. The titre of the control calf commenced between 5 and 7 weeks after this exposure and 2 weeks after larvae appeared in the faeces. Mild symptoms developed in these calves and the infection was patent for about 4 weeks in the vaccinates and 10 weeks in the control (Table 2).

Fig. 8



Antibody titre of calf 212.
V = dose of vaccine given. O = turned into orchard.
G = transferred to heavily infected pasture at Field Station. D = died.

Experiment 5. Calf 212 (Fig. 8) vaccinated with an interval of 6 weeks went into the orchard for 7 weeks with the calves in Experiment 4. It was then taken to the Field Station and exposed on a heavily infected pasture. This calf had developed only a transient titre to vaccination and the titre was zero when it was exposed to the heavily infected pasture which had proved lethal to two unvaccinated calves. The titre rose within 7 days and reached 320 in 3 weeks, but the calf died from acute husk before the infection could become patent and approx. 15,000 worms were present in the lungs.

TABLE 3

Calf	*Degree of patency (highest faecal larval count)*	*Titre at challenge*	*Titre 2 weeks after challenge*
215	1.3 l/g	80 — 60	60 — 30
216	1.7	10	40
184	1.7	80	80 — 40
125	1.9	80	160
217	2.7	60	160
073	4.4	20	60
240	7.9	40	80
938	27.0	0	20
239	131.0	0	15
538	212.0	10	5
212	—	0	30 — 320
937 Controls	17.0	0	0
134 Controls	42.7	0	0

DISCUSSION

The majority of vaccinated calves exposed to challenge showed a titre which continued to rise as a result of stimulation by vaccination. In those cases where no response to vaccination was evident an early rise in titre followed the challenge exposure, indicating that sensitisation had taken place. In contrast, the control calves showed a delay of 5 to 6 weeks between exposure and the commencement of antibody production as found with other outdoor calves (Cornwell and Michel, 1960). The similarity between the mean titre of the vaccinated calves and the titre of the control calf in Fig. 1 is striking and would suggest that the antigenic stimulus provided by vaccination in these calves was similar to that experienced by the control calf on infected pasture. In all calves the titre after reaching the peak fell gradually until removal from the pasture when a sharper fall was evident. This behaviour is also typical of unvaccinated calves exposed to infected pasture (Cornwell and Michel, 1960).

It was possible to estimate the degree of herbage infestation experienced by calves 125 and 240 at the Field Station (Figs. 6 and 7) and it was found that the maximum titres occurred at the time the pasture level was at its height and when the calves were still excreting larvae. The titres fell on returning to the sparse grazing on the farm and continued to fall after housing.

Although calves 073 and 239 had markedly different titres up to the time of challenge with 10,000 larvae, there was afterwards a close similarity. This suggests that the response to vaccination in the two calves differed only in quantity, both of them showing evidence of previous contact with the parasite by producing a peak response to the challenge in 3 weeks. In the absence of further exposure the titres fell, but a secondary response was elicited by turning the calves onto an infected pasture. Of these two calves 239 was less resistant and experienced two periods of patency resulting from the two challenges. There may be some connection here between the absence of a demonstrable response to vaccination and this calf's relative susceptibility to challenge. Reference to Fig. 4 will show that the two peaks of antibody production which followed the two challenges in these calves, coincided with an increase in respiratory rate probably indicating that worms were developing in the lungs. This supports the suggestion made by Cornwell and Michel (1960) that the secondary response is due to the worms of a challenge dose becoming established in the lungs of an animal which has had previous experience of infection.

Calf 212 (Fig. 8) showed no evidence of resistance to a heavy field infection and died from acute husk in the prepatent stage. Its titre showed only a transient response to vaccination and was zero at the time of challenge. A good secondary response was evident to the challenge. Evidently the immunity afforded by vaccination is not sufficient to protect against a sudden heavy acquisition of infection. In the case of this calf the vaccine had obviously "taken" in some degree as shown by the nature of the antibody response to the challenge.

Calf 538 (Table 1) developed only a very low titre after vaccination and the second dose did not "boost" the titre. The challenge of the infected pasture produced a rise in titre intermediate between a primary and secondary response and when it died the titre was 160, the faecal larval output was 212 per gramme and 860 worms were present in the lungs. It appears that in vaccinated calves the antibody response to challenge develops early and reaches a peak before the faecal larval output attains its maximum. This is the reverse of the picture given by unvaccinated calves receiving an initial infection (Jarrett *et al.* 1959; Cornwell and Michel, 1960), but similar to that shown when they are reinfected, except that they seldom show more than a few larvae in the faeces resulting from the challenge.

It thus appears that the effect of vaccination is to imitate a mild natural infection. On challenge the calf shows evidence of this both by the degree of immunity shown and by a continued rise in antibody level or its early onset. The immunity afforded by vaccination may be similar in degree to that produced by a light natural infection in unvaccinated calves and it is not surprising that a challenge infection should produce a low degree of patency in the majority of animals. Occasionally, however, a vaccinal immunity is of a low order and the degree of patency resulting from challenge is high.

The explanation of the differing response to vaccination, both in titre and resistance to challenge, may lie in the differing degree to which the irradiated larvae migrate in the body. Jarrett *et al.* (1957) have suggested that the site of the primary immune stimulus is the mesenteric lymph node, although they have reported that some of the irradiated larvae succeed in reaching the lungs producing a transient rise in respiratory rate but minimal lesions (Jarrett *et al.*, 1959). It seems likely that the degree to which irradiated larvae succeed in developing in the lungs will vary from calf to calf, and this may well be linked with the variable antibody response to vaccination. If this is so then the resistance of vaccinated calves may depend on the degree to which the irradiated larvae become established in the lungs and if the development of a titre is an indication of this, it should be possible to demonstrate a connection between a low vaccinal titre and low resistance. An attempt to do this has been made in Table 3, where the degree of patency, reckoned as the highest faecal larval count of those calves whose faeces were examined regularly, is compared to the titre at the time of challenge and 2 weeks later. Two control calves are included for comparison. It will be seen that the calves which developed the highest degree of patency had low or zero titres at the time of challenge and that their response was still low two weeks later. The calves showing a low degree of patency had titres of 10 to 80 at challenge and 40 to 160 two weeks later. The degree of patency of calf 212 is not known, but judging from the numbers of young gravid females in the lungs, it would have been high. It is difficult to draw definite conclusions from these figures because so much depends on the challenge met in each case, but there does seem to be a tendency for the less resistant calves to have a low titre at challenge and only a small rise in the two weeks afterwards. In this respect they occupy a place intermediate between the control calves which have a delayed titre and the more resistant ones which already have a well developed titre at challenge or show a good response within the 2 weeks.

Although the antibody titre being measured here gives no true indication of an animals' ability to resist re-infection it must be the result of the interaction of parasite and host, and probably runs a parallel course at some stages of the infection with the protective antibodies which are known to occur but have not yet been demonstrated.

CONCLUSIONS

The antibody response of 19 calves vaccinated with a double dose of irradiated *D. viviparus* larvae and subsequently exposed to challenge was measured by the complement fixation test using a heated whole worm antigen. The challenge stimulated an early rise in titre or a continuation of the rise produced by vaccination. In the calves challenged at grass, the titre reached a peak and continued high as long as the pasture remained infected, but commenced to fall at the end of the season when the pastures were bare, and continued to fall after housing.

In two calves challenged indoors with 10,000 larvae the titre rose to a peak and fell almost as quickly. Later exposure at grass stimulated a secondary response. These peaks of antibody response coincided with a rise in respiratory rate.

An assessment of the effectiveness of the vaccine was made. One calf exposed to a very heavy field infection died in the prepatent stage although its titre showed a marked secondary response. The two calves challenged indoors with 10,000 larvae exhibited moderate symptoms and loss in weight occurred. Calves exposed to an estimated moderate degree of infection at pasture showed mild symptoms and small numbers of larvae were passed in the faeces. One exception occurred in this group, the calf dying while passing 212 larvae per gramme and having 860 worms in the lungs.

There is some evidence to show that those calves whose vaccinal titres are low or absent and do not rise as quickly as in a true secondary response are less resistant to challenge.

It is suggested that the development of immunity depends upon the establishment of irradiated larvae in the lungs.

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